REMARKS

In the Office Action dated May 23, 2005, Claims 1-10 are pending. Claims 11-15 have been withdrawn as directed to non-elected subject matter. New claims 16-17 have been added. Claims 1-10 have been rejected under 35 U.S.C. §103(a) as allegedly unpatentable over U.S. Patent No. 4,676,982 to Hassig ("Hassig"), in view of U.S. Patent No. 4,477,432 to Hardie ("Hardie"), and further in view of Park et al. (*Arthritis and Rheumatism*, vol 37, p. R5, 1994) ("Park et al.") and Ibbotson et al. (*Gut*, vol 36, p. 1-4, 1995) ("Ibbotson et al."). The Examiner has requested copies of certain references relied upon herein.

Applicants have added claims 16-17 directed to a method of treating ulcerative colitis and Crohn's disease, respectively. Support for claims 16-17 is found throughout the specification and specifically at page 4, lines 5-6, claim 2 and Example 1. No new matter has been added.

Claims 1-10, have been rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Hassig, in view of Hardie, and further in view of Park et al. and Ibbotson et al. The Examiner alleges that the primary reference to Hassig teaches a method of treating certain IBD diseases by intravenously administering a pooled human immunoglobulin preparation. The Examiner alleges that the secondary reference to Hardie discloses a method for treating enteric infection by orally administered immunoglobulin; and that oral administration of immunoglobulin has certain advantages over intravenous administration (i.e., to avoid pain from an injection). The Examiner alleges that Park et al. teach the treatment of rheumatoid arthritis (RA) by orally administering pooled human immunoglobulin to neutralize superantigens related to the RA pathogenesis. The Examiner further alleges that Ibbotson et al. suggest that superantigens are involved in the pathogenesis of IBD.

Thus, the Examiner contends that one skilled in the art at the critical time would have been motivated to substitute the "advantageous" oral administration of immunoglobulin taught by Hardie for the intravenous administration of immunoglobulin taught by Hassig. The Examiner contends that considering that Park et al. suggest that pooled human immunoglobulin may neutralize superantigens related to the pathogenesis of RA, and further considering that Ibbotson et al. suggest that superantigens may be involved in the pathogenesis of IBD, one skilled in the art at the critical time would have been motivated to treat IBD with orally administered pooled human immunoglobulin.

Applicants respectfully submit that the Examiner's proposition fails to establish a *prima facie* case of obviousness. At best, the Examiner's proposal establishes only that it would have been "obvious to try" the asserted combination, without a reasonable expectation of success. Notably, the Examiner's analysis employs considerable impermissible hindsight; and the motivation to combine the references is not properly found in the cited art, absent reference to the present invention.

Applicants submit that the present invention, for the first time, surprisingly identifies that oral administration of pooled human immunoglobulin is therapeutically effective for treating IBD.

Applicants emphasize that the etiology of IBD remains unknown. Hence, a combination of prior art that is premised upon a certain alleged "etiology" of IBD is ultimately based on speculation and cannot support an assertion of obviousness. Applicants observe, for example, that the primary reference to Hassig acknowledges, in the first instance, that "[c]ertain inflammatory conditions of the bowel are of unknown etiology and are difficult to treat." Hassig, col. 1, lines 9-10 (emphasis added). Hassig further specifies ulcerative colitis (col. 1,

line 11) and Crohn's disease (col. 1, line 13) as known IBD diseases, which are of "unknown etiology."Although Hassig teaches intravenous administration of immunoglobulin in the treatment of IBD, Applicants respectfully submit, as admitted by the Examiner, that there is, however, no recognition in Hassig, or any other reference cited on this record, that oral administration of pooled human immunoglobulin would be therapeutically effective for treating IBD.

Notably, the cited secondary references, taken separately or in combination, do not ameliorate the deficiencies of Hassig.

A rejection of claimed subject matter as obvious under 35 U.S.C. §103 requires consideration of two factors: (1) whether the prior art would have suggested to those of ordinary skill in the art that they should make the claimed device or composition, or carry out the claimed process; and (2) whether the prior art would have suggested that in so carrying out the claimed process, those of ordinary skill would have a reasonable expectation of success. *In re Vaeck*, 947 F.2d 488, 493, 20 U.S.P.Q.2d 1438, 1442 (Fed. Cir. 1991). There is no suggestion in the art of record to treat IBD by orally administering pooled human polyclonal immunoglobulin, as presently claimed. Nor is there any expectation in the prior art on this record that oral administration of pooled human immunoglobulin can in fact treat IBD.

Hardie is directed to the use of orally administered immunoglobulin in treating enteric infections, e.g., infections caused by *E. coli, V. cholera,* or *S. typhosa;* or in treating intoxications, e.g., infantile botulism, (col. 7, lines 3-10 of Hardie). Applicants observe that enteric infections are diseases that, unlike IBD, have known etiologies, e.g., are caused by bacteria or viruses. Hardie does not teach or suggest the treatment of any inflammatory disease, let alone IBD.

Hardie also discloses that intact human immunoglobulin, when administered orally, would not lose its therapeutic efficacy. According to Hardie, the therapeutically effective immunoglobulin is delivered (via oral administration) to the site where the therapeutic activity of the immunoglobulin is required. However, nowhere does Hardie teach or suggest that treating IBD requires the therapeutic activity of immunoglobulin in the gastrointestinal tract. In fact, Hardie discloses that orally administered immunoglobulin "may be used in prevention or treatment of enteric infections . . . since intact IgG with opsonic activity persisted in the gastrointestinal tract and thus is available to function in such prevention or treatment." See, Hardie, col. 7, lines 3-8 (emphasis added).

Applicants submit that it is well known in the art that the opsonic activity of IgG involves guiding phagocytic cells to ingest and destroy the infection-causing bacteria by coating such bacteria with IgG that would be recognized by the phagocytic cells. Thus, Hardie teaches, at most, that if retained intact in the gut, immunoglobulin is useful for treating enteric infection. One may not, however, extrapolate the therapeutic activity of intact immunoglobulin in treating enteric infection to treating all other diseases affecting the gastrointestinal tract.

Moreover, Applicants observe that the intestinal infections in Hardie merely involve immature infants. Applicants submit that the gastrointestinal tract of an infant is physiologically and biochemically distinct from that of an adult or a child, which carries a mature digestive system that may readily degrade immunoglobulin.

Hardie assumes that to be effective, immunoglobulin molecules must survive the gastrointestinal environment to reach their target areas with intact biological properties. However, there is no teaching or suggestion in Hardie that orally administered immunoglobulin can survive the gastrointestinal environment in adults and children. To combine Hassig and

Hardie to achieve the present invention, one must assume that immunoglobulin would also survive the gastrointestinal environment in adults and children, which assumption is both unwarranted and unfounded in the references on this record. In fact, Hassig clearly acknowledges that immunoglobulin may be cleaved or degraded by pepsin (a well-known enzyme that exists at high concentration in mature digestive systems). See Hassig, col. 1, lines 32-35. Alternatively, one must assume that degraded immunoglobulin can still function for treating IBD. Such assumption is flatly contradicted by the disclosures of Hassig and Hardie, which explicitly require intact immunoglobulin for therapeutic efficacy. Notably, the discovery of the present invention is not premised on the ground that immunoglobulin remains completly intact in the gut in the first place.

Furthermore, even assuming immunoglobulin would have remained intact in the gut of adults and children, one skilled in the art could only guess as to whether or not the intact immunoglobulinin the gastrointestinal tract could function to treat IBD. Moreover, absent the teachings of the present invention, the result of treating IBD by oral administration of pooled human immunoglobulin would be, at best, unpredictable.

Accordingly, Hardie merely teaches that molecular integrity may be necessary for immunoglobulin 's therapeutic efficacy for treating enteric infections. Hardie does not teach or suggest that oral administration of intact immunoglobulin is either necessary or sufficient for treating IBD.

Thus, Hardie does not ameliorate the deficiencies of Hassig as to whether oral administration of immunoglobulin can treat IBD.

The Park et al. reference is a one-paragraph meeting abstract in which certain beneficial effects resulting from the oral administration of immunoglobulin to RA patients in a 6-

week study is disclosed. Nowhere do Park et al. disclose or suggest a method for treating IBD. Even to the extent of treating RA, Park et al. admit that the reported results are from a "limited period of observation" and merely "encourage further evaluation" of oral immunoglobulin in the treatment of RA. Park et al. further speculate that the neutralization of superantigens by immunoglobulin may be implicated in the treatment of RA as disclosed, or that such mechanism could explain these initial observations. Careful reading of such disclosure, in the absence of the present invention, however, provides merely an invitation to further experimentation. Park et al. do not remotely suggest, as the Examiner asserts, that RA or any autoimmune disease, particularly a disease of unknown etiology, such as IBD, is in fact caused by superantigens; or could be addressed on that basis.

The Examiner also relies on Ibbotson et al. to support the proposition that IBD is caused by superantigens. Applicants observe that the Ibbotson et al. reference is not a research article reporting original discoveries, but merely a review article discussing the potential role of superantigens in the pathogenesis of Crohn's disease. (See the title and the paragraphs starting under the last heading on page 2, right column (emphasis added)). Applicants observe that the last two paragraphs of Ibbotson et al., upon which the Examiner relied in the Office Action, merely propose a possible factor among many others allegedly involved in the etiologies of IBD. In this connection, Applicants provide copies of several references upon which Applicants observe that numerous clinical, epidemiological, and experimental studies have been conducted in an attempt to elucidate the etiology of IBD (for example, Fiocchi, *Gastroenterol.* 1998; 115:182-205, attached hereto as Exhibit A, Banic et al. *Acta Med Croatica* 1997;51(1):37-40), attached hereto as Exhibit B). A number of factors, individually or in combination, have been suggested or suspected as possible causative agents for IBD, e.g., genetic factors (McLeod et al.

Dis Colon Rectum 1997;40(5):553-7, attached hereto as Exhibit C), microbial factors (Stable *J Dairy Sci* 1998;81(1):283-8, attached hereto as Exhibit D), viral factors (Smith and Wakefield *Ann Med* 1993;25(6)557-61, attached hereto as Exhibit E), immunological factors (Beil et al. *J Leukoc Biol* 1995;58(3):284-98, attached hereto as Exhibit F), nutritional factors (Bielefeldt et al. *Gastroenterol* 1989;27(9):455-8, attached hereto as Exhibit G), thrombotic factors (Levine et al. *J Pediatr Gastroenterol Nutr* 1998;26(2):172-4, attached hereto as Exhibit H) and environmental factors (Koutroubakis et al. *Hepatogastroenterology* 1996;43(8):381-93, attached hereto as Exhibit I). Applicants respectfully submit that in spite of the many suspected factors or mechanisms, as illustrated above, and the increasing interest in treating IBD, none of the studies have been able to demonstrate a reasonably clear cause and effect relationship and the etiology of IBD remains unknown. See the webpage (http://people.hsc.edu/faculty-staff/edwardd/edsweb01, attached hereto as Exhibit J) of Dr. Edward W. Devlin at Hampden-Sydney College, Hampden-Sydney, Virginia (emphasis added).

Notably, Ibbotson et al. in fact clearly state that the etiology of IBD "remains unknown" (the first paragraph of the article) and "searches for evidence of autoimmune reactions in IBD, especially Crohn's disease, have been negative" (the first full paragraph on page 3, left column).

Thus, Applicants respectfully submit that in the context of a disorder with still unknown etiology and no viable treatment, the collective wisdom of the art at the relevant time provide no more than an invitation to experiment which satisfies only an "obvious to try" standard, long rejected under the law. *Ex parte Goldgaber*, 41 USPQ 2d 1172, 1177 (B.P.A.I. 1996) (quoting *In re Eli Lilly and Co.*, 902 F.2d 943, 945, 14 USPQ 2d 1741, 1743 (Fed. Cir. 1990)).

Even assuming, *arguendo*, that one skilled in the art was motivated to combine the teachings of Hassig and the secondary references at the time the present application was filed, there would have been no reasonable expectation of success to achieve the present invention. As discussed above, to combine the cited art in the manner the Examiner has asserted, one skilled in the art at the relevant time would have to speculate as to whether immunoglobulin survives adult and children's digestive system; whether immunoglobulin in the gut in fact functions for treating IBD; or whether IBD is in fact caused by superantigens. Based on these speculations, absent the teachings of the present invention, there would have been simply no reasonable expectation of success for one skilled in the art to achieve the present invention, i.e., treating IBD by oral administration of pooled human immunoglobulin.

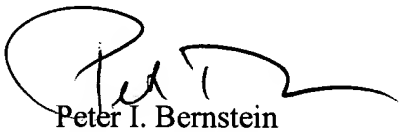
Moreover, there had been an increasing interest and a long-felt need for an effective treatment method for IBD at the time the present invention was filed. See, e.g., *supra* website Dr. Devlin and the specification, page 2, line 15 to page 5, line 7. The present invention provides a successful solution to this long-standing problem. The hypothetical combination of the cited references is not likely to defeat an invention where the evidence shows that long-standing problems were solved. *Kalman v. Kimberly-Cark Corp.*, 713 F.2d 760, 774, 218 U.S.P.Q. 781, 791 (Fed. Cir. 1995). Long-felt need in the face of prior art later asserted to lead to a solution tends to negate the proposition that the combination of such prior art would have been obvious. *Micro Chem., Inc. v. Great Plains Chem. Co.*, 103 F.3d 1538, 1547, 41 U.S.P.Q.2d, 1238, 1245 (Fed. Cir. 1997) (emphasis added). Thus, by solving a long-standing problem, the present invention is not obvious in view of the cited art.

Furthermore, Applicants submit that the Examiner's combination of Hassig, Hardie, Park et al. and Ibbotson et al. can, in fact, only be made with the benefit of hindsight,

derived from the disclosure of the present application. Applicants submit that the rejection of claimed subject matter under 35 U.S.C. §103 requires that both the suggestion to carry out the claimed invention and the reasonable expectation of success must be found in the prior art, not in Applicants' disclosure. *In re Vaeck*, 947 F.2d 488, 493, 20 U.S.P.Q. 1438, 1442 (Fed. Cir. 1991) (emphasis added). Here, the suggestion and the expectation of success of a method for treating IBD by oral administration of pooled human immunoglobulin preparation appears nowhere in the cited art, and only in the present application.

In view of the above remarks, it is respectfully submitted that the present invention is not-obvious in view of Hassig, Hardie, Park et al. and Ibbotson et al. Accordingly, the rejection of Claims 1-10, under 35 U.S.C. §103(a) is overcome and withdrawal thereof is respectfully requested.

Respectfully submitted,



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GASTROENTEROLOGY 1998;115:182–205

SPECIAL REPORTS AND REVIEWS

Inflammatory Bowel Disease: Etiology and Pathogenesis

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The field of gastroenterology offers many challenges to both the clinician and the investigator, but few are as complex and enigmatic as inflammatory bowel disease (IBD). Crohn's disease (CD) and ulcerative colitis (UC) have been known for well over one half a century, but why affected individuals spend their lives with a chronic inflammatory process that relentlessly destroys their bowel remains a mystery. Advances, although unequal in different areas, have been plentiful in the last decade when the combined power of cellular and molecular biology began to unveil the enigmas of IBD. No single agent or distinct mechanism is the sine qua non motive that explains all aspects of IBD, and several distinguishing factors are likely necessary to result in either CD or UC (Table 1). This review will attempt to discuss those that currently appear important.

Conditioning IBD: Environment and Genes

Environmental Factors

Among the many puzzles of IBD pathogenesis, one of the less understood and most difficult to tackle is the role of environmental factors in the appearance and progression of CD and UC in this century. Environmental factors are probably as important as the patient's genetic makeup for the risk of IBD. Potentially relevant environmental factors include prenatal events, breastfeeding, childhood infections, microbial agents, smoking, oral contraceptives, diet, hygiene, occupation, education, climate, pollution, stress, and miscellaneous components such as toothpaste, appendectomy, tonsillectomy, blood transfusions, contact with animals, and physical activity.[1] Among these, the most established association is with smoking, but the reproducibility and strength of association with other risk factors needs further investigation.

Smoking. The effect of smoking or, curiously, the opposite effect of smoking on the outcome of each form of IBD represents the most intriguing connection between environmental factors and IBD. With one exception, reports have shown that nonsmoking is a feature of patients with UC, whereas smoking is a feature of patients with CD.[2–4] Smoking is an independent risk factor for clinical, surgical, and endoscopic recurrence in CD and influences disease activity after surgery. Although the components of tobacco responsible for these observations are uncertain, transdermal nicotine patches added to conventional therapy improve symptoms in patients with mild to moderate UC.[5] A modulatory effect of nicotine on immune responses in vitro has been observed.[6] However, what relationship, if any, exists between these in vitro responses and the in vivo effect on IBD is unclear. The inverse association in UC and CD is especially puzzling and may be linked to differences in the pathogenesis of the two disorders.

Diet. A link between diet and IBD is logical because IBD affects the very site of nutrient absorption. Nutritional deficiencies in IBD are well documented, particularly that of zinc in CD with associated immunologic dysfunction.[7] The effectiveness of elemental or special diets in reducing the symptoms or inducing remission of CD has been proposed but not universally accepted, and some studies have found that enteral nutrition is less effective than steroids and aminosalicylates.[8,9] Moreover, the relapse rate does not appear to differ between nutritional and traditional approaches,[10] even when exclusion diets are implemented.[11] Some data suggest that elemental diet may improve CD by reducing intestinal permeability,[12] but it is not clear why nutritional therapies improve CD but not UC.

Intestinal permeability. Increased intestinal permeability may play a role in the pathogenesis of patients with CD. In addition to the patients themselves, increased permeability can be found in symptom-free first-degree relatives.[13] Several studies have confirmed this observation, although results depend on the method-

Abbreviations used in this paper: COX, cyclooxygenase; ICAM-1, intercellular adhesion molecule 1; IFN-γ, interferon gamma; IL, interleukin; PAF, platelet-activating factor; pANCA, perinuclear antineutrophil cytoplasmic antibody; PMN, polymorphonuclear leukocyte; ROM, reactive oxygen metabolite; TGF, transforming growth factor; TNF, tumor necrosis factor.

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Table 1. Distinguishing Features of UC and CD Pathogenesis

Component	UC	CD
Environmental factors	Beneficial effect of smoking	Detrimental effect of smoking
	No beneficial effect of diet	Symptoms improved by selected diets
	Normal intestinal permeability in healthy relatives	Increased intestinal permeability in healthy relatives
Genetic associations	Largely different from CD	Largely different from UC
Microbial agents	Limited role of bacterial flora	Important role of bacterial flora
	No association with *M. paratuberculosis*	Association with *M. paratuberculosis*
	No association with measles virus	Some association with measles virus
Humoral immunity	Prominent antibody secretion	Moderate antibody secretion
	Evidence for autoimmunity	Limited evidence for autoimmunity
	Strong association with ANCA	Weak association with ANCA
Cell-mediated immunity	Prominent neutrophil infiltration in the mucosa	Prominent T-cell infiltration in the mucosa
		Hyperreactive T cells
	Normal/hyporeactive T cells	Resistance of T cells to apoptosis (?)
	Normal T-cell apoptosis (?)	
Cytokines and mediators	Prominent production of eicosanoids	Moderate production of eicosanoids
	Th2-like profile	Th1-like profile
	Increased cytokine production limited to involved mucosa	Increased cytokine production in involved and uninvolved mucosa

ANCA, antineutrophil cytoplasmic antibodies.

ology used, and some have failed to find altered permeability in relatives. Determining whether first-degree healthy relatives of patients with CD have increased permeability is of considerable pathogenic importance because it could represent a predisposing factor leading to overt inflammation and clinical manifestations later in life. Of note, increased intestinal permeability antedating clinical manifestations in CD has been reported.[14] Although permeability is normal in the majority of relatives, those with a high permeability rate may represent a subgroup prone to developing CD.[15] Indeed, when first-degree relatives are challenged with acetylsalicylic acid, permeability increases in some of them.[16] The possible consequences of abnormal permeability have been shown by the fact that relatives have a concomitant increase in the number of circulating $CD45RO^+$ B cells. This suggests that a leaky intestinal barrier intensifies antigen absorption, which, in turn, leads to an exaggerated immune stimulation reflected at the systemic level by an excessive number of mature B cells.[17] If correct, this finding would provide a direct link between an altered intestinal barrier and the well-documented immune aberrations of CD.

Colonic mucus. Mucus represents a major component of intestinal defenses, and defects of this tough layer of glycoconjugates all along the gastrointestinal tract could predispose to IBD, UC in particular. A selective alteration of colonic mucin composition in involved and uninvolved mucosa of patients with UC but not CD was proposed[18] but subsequently challenged by studies showing that the composition of purified mucins in UC and control subjects was similar.[19] These discrepancies could be ascribed to methodological differences, but even if colonic mucin alterations were present, they alone would be insufficient to trigger inflammation because twins with the same defect are discordant for the occurrence of UC.[20]

Familial and Genetic Factors

Family studies. One of the most consistent observations in studies of IBD populations is the high incidence of CD or UC among family members of the index case. Both vertical and horizontal associations occur, including father-son, father-daughter, mother-son, mother-daughter, and sibling-sibling. The frequency of IBD in first-degree family members may be as high as 40%.[21] Both genetic and environmental factors have been considered as the basis for these associations. Discriminating between the two has been difficult because data can be interpreted in different ways. A large international cooperative study found very few statistically significant differences between index and control families.[22] Except for increased incidence of eczema, no other differences were found between patients and controls in regard to a multitude of factors traditionally linked to IBD, including breastfeeding, childhood gastroenteritis, sugar and cereal consumption, and stressful events of life, among others. In large studies performed in Scandinavian countries, a 10-fold increase in familial risk was interpreted to be strongly suggestive of a genetic cause,[23] whereas seasonality of the cohort effect combined with the urban preponderance was interpreted as pointing to environmental factors.[24] A higher rate of concordance exists for monozygotic twins with CD or UC compared with dizygotic twins.[25] Immunologic abnormalities such as higher frequency of antibodies to *Escherichia coli* O:14 antigens and intestinal epithelial antigens in healthy first-degree relatives of patients with IBD have also been reported.[26,27] However, these observations do not permit a clear distinction between genetic and environmental contribution. Of note, the frequency of affected first-degree relatives tends to be higher in CD than in UC. On the other hand, there is usually concordance of clinical

characteristics between the index case and family members in regard to age, site of inflammation, and type of clinical manifestations.[28] At the very least, it is clear that neither form of IBD can be explained by a simple mendelian inheritance pattern.

Genetic studies. In parallel with the above clinical observations, many studies investigating genetic factors in IBD have been performed (Table 2). An early investigation in a random European population found a significantly increased frequency of HLA-A11 and HLA-A7 in UC and a decreased frequency of HLA-A9 in CD.[29] One of the most reproducible associations in a defined population is that with HLA-DR2 in Japanese subjects with UC.[30] In contrast, no DR2 association is observed in British and American patients with IBD, although association with DRB1*103 and DRB1*12 is found in UC, including negative and positive associations with extent and severity of disease and extraintestinal manifestations.[31–33] These differences show the value of less heterogeneous groups, such as the Japanese population, to minimize contrasting results among ethnically and genetically diverse populations in various countries. There is good evidence for genetic heterogeneity in IBD, which is probably responsible for disparate associations with class II antigens, such as positive association with the HLA-DR2 and a negative association of DR4 and DRw6 in UC and a positive association with DR1 and DQw5 in CD.[34] Studies combining genetic and immunologic markers corroborate the existence of genetic heterogeneity in IBD,[35] but the consistency of findings varies depending on the target population.[32]

Table 2. Genetic and Immunologic Associations Reported in IBD

Association	UC	CD
HLA-A3	Decreased	None
HLA-A9, HLA-B27		Decreased
HLA-A7, A11	Increased	None
HLA-B12, DR1, DQB1*0501		Increased[b]
HLA-Bw52, -Bw35, DQw1, DPB1*0901	Increased[a]	
DR2, DRB1*1502	None, increased[a,b]	None
DR4, DRw6	Decreased[a,b]	None
DRB1*01, *07, *0501, *1302, DRB3*0301		Increased
DRB1*03, DQB1*0602, *0603		Decreased
DR3, DQ2	Extensive disease, decreased in women	
DRB1*0103	Extensive disease, extraintestinal manifestations	
Chromosomes 3, 7, and 12	Linkage	Linkage
Chromosome 6		No linkage
Chromosome 16	No linkage	Linkage[c]
ANCA-positive	Increased	
IL-1 receptor antagonist allele 2	Extensive disease	
$TNFa_2b1c2d4e1$	None	Increased
TNF-α-308 allele 2	Decreased	No linkage
ICAM-1, ANCA-negative	Increased	
ICAM-1, ANCA-positive		Increased

ANCA, antineutrophil cytoplasmic antibodies.
[a]Japanese patients.
[b]Mostly Jewish patients.
[c]No linkage in Jewish patients.

Evaluation of Japanese patients with CD found susceptibility and resistance linked to DQB1*4 and DQA1*0102, respectively.[36] In a large cohort of French patients with CD, however, susceptibility was associated with the HLA-DRB1*01 and DRB1*07 alleles, and resistance was associated with the DRB1*03 allele,[37] whereas an increased risk for CD, but not UC, was detected in an American white population carrying the HLA-DRB3*0301 allele.[38] In sharp contrast, no evidence that class II genes contribute to CD susceptibility and disease phenotype was reported in a British study.[31] Associations of IBD with interleukin (IL) 1 receptor antagonist, intercellular adhesion molecule 1 (ICAM-1), and tumor necrosis factor (TNF)-α genes have been described, but the strength of these associations is variable and generally weak.[39–42] Using large aggregates of families with IBD, recent studies at the genomic level started to identify linkages to specific chromosomes. A French group reported that CD genetic predisposition resides outside of chromosome 6[43] and subsequently reported a susceptibility locus on chromosome 16,[44] a finding confirmed by other investigators. A British group reported evidence for susceptibility for both CD and UC on chromosomes 3, 7, and 12, suggesting that these are distinct disorders sharing some, but not all, susceptibility genes.[45] These observations not only provide new insights into potential IBD genes but may help discard older and inconsistent data obtained with flawed serological methods.

A concept that further supports the relevance of genes to disease predisposition is that of genetic anticipation, defined by a progressively earlier disease onset in successive generations.[46] Multicenter screening studies using well-defined groups of patients and families will undoubtedly provide invaluable information on the role of genetics in IBD. In the midst of new enthusiasm, it should not be forgotten that even if identical twins are significantly more likely to develop IBD than nonidentical twins, this only accounts for part of the concordance. Furthermore, there is an irreconcilable disparity between the rapid increase in IBD incidence during the last few decades and any plausible changes in the genetic makeup of stable populations during the same period of time. Thus, nongenetic, i.e., environmental factors, are clearly

indispensable to develop IBD through a still nebulous interaction with multiple predisposing, but not sanctioning, genes.

Causing and Promoting IBD: Microbial Agents

Bacteria

An infectious etiology for IBD, with a direct cause-and-effect relationship between a single microorganism and inflammation, still remains plausible. The relationship between microbes and defined clinical entities is often ambiguous, but diseases of unknown etiology are unexpectedly proven to be infectious. The most striking example is peptic ulcer disease and *Helicobacter pylori*. Although traditional methods have failed to detect a specific pathogen associated with IBD, newer molecular biological guidelines are now available to establish microbial causation in IBD.[47]

Immune reactivity to microbial antigens. A traditional approach to identify microbial agents linked to IBD has been the demonstration of antibodies against known microorganisms. Essentially all patients with IBD have elevated titers against bacteria, viruses, and fungi, making the mere presence of serum or intestinal antibodies of limited value. Although patients with active CD have elevated serum antibody titers to multiple enteric bacterial pathogens,[48] this seroreactivity likely represents a secondary sensitization to cross-reacting antigens or a nonspecific enhancement of immune reactivity.

Cell-mediated immunity against microbes has also been assessed. Early studies showed that migration of leukocytes in vitro was inhibited by colonic homogenates in UC but not CD, and subsequent studies showed migration-inhibitory activity against enterobacterial common antigen by peripheral blood lymphocytes of patients with IBD.[49] These results should be interpreted in the context of "physiological intestinal inflammation," implicit to which is tolerance to local endogenous bacteria. Thus, although T cells from uninvolved mucosa fail to proliferate in vitro on stimulation by pathogenic and nonpathogenic microbial antigens in CD, the heightened response of T cells from involved mucosa implies antigen-specific recognition and loss of tolerance.[50]

Intestinal flora. The possibility that components of the normal intestinal flora could trigger, initiate, or somehow contribute to IBD has intrigued investigators for years. Differences in microbial ecology among individuals with UC and CD and normal individuals were not strongly supported by early investigations, but a recent study found a significant decrease in the number of anaerobic bacteria and *Lactobacillus* in patients with active but not inactive IBD.[51] It is possible that products of the commensal flora promote inflammation in the presence of an impaired mucosal barrier or injury to the mucosa.[52] The concept that the normal flora somehow functions as a modulator of "physiological inflammation" has been strengthened substantially by the observations of Duchmann et al.[53,54] They have shown that mucosal, but not peripheral blood, mononuclear cells from patients with IBD proliferate when exposed to autologous intestinal bacteria. Cells from uninvolved mucosa of the same patients and from patients in remission failed to proliferate to autologous IBD flora. Blood and mucosal mononuclear cells from normal individuals, however, fail to proliferate to autologous bacteria, but do so in response to sonicates of bacteria from heterologous intestine. These results indicate that, in health, there is tolerance to autologous but not allogeneic intestinal flora, and tolerance is lost during inflammation.[53] Evidence also exists that animals are tolerant to their own flora in health but not after colitis develops.[54] Infection breaks T-cell tolerance, and the hypothesis that intestinal infections abrogate tolerance leading to a chronic immune response to self flora in susceptible individuals is appealing.

Fecal stream and bacterial stasis. The concept that intestinal contents contribute to IBD pathogenesis is gaining momentum. Fecal stream diversion may determine recurrence of CD.[55] Patients with ileal resections and a diverting ileostomy excluding the neoterminal ileum fail to develop recurrent disease until reanastomosis is performed. Furthermore, infusion of autologous luminal contents in excluded normal ileal loops of patients with CD rapidly induces new inflammation, indicating that fecal component(s) may promote disease flare-up.[56] These observations are reminiscent of pouchitis, where repopulation of the pouch by enteric flora seems to be essential for induction or persistence of inflammation.[57] The common denominator of all these observations is a change in the quantity or quality of bacterial and dietary luminal content. In animals, excessive enteric bacteria lead to increased expression of cell adhesion molecules and granulocyte infiltration, and both are reduced by antibiotics or speeding fecal flow.[58] These clinical and experimental observations suggest that microbial imbalance could be a major factor in the development or maintenance of chronic intestinal inflammation.

Mycobacteria. An association of CD with mycobacteria has been postulated since the original description of terminal ileitis. This idea has been resurrected over a decade ago by the recovery of an unclassified acid-fast organism related to *Mycobacterium paratuberculosis* from CD tissues.[59] Neonate goats fed with such an organism

showed evidence of both humoral and cellular immunity to it and developed granulomatous inflammation in the terminal ileum. This observation spurred a tremendous amount of investigation followed by very inconsistent results. Some showed that patients with CD had significantly increased serum antibody titers to *M. paratuberculosis,*[60] but subsequent studies failed to find a significant elevation of serum immunoglobulin (Ig) A, IgG, or IgM to mycobacterial antigens,[61] despite evidence of contact with other environmental mycobacteria. No evidence of systemic or mucosal cell-mediated immunity to *M. paratuberculosis* has yet been shown.

Detecting *M. paratuberculosis* in involved tissues has been difficult. Immunohistochemistry fails to show the organism in tissue sections,[62] and isolation of *M. paratuberculosis* in bacterial or spheroplastic form has met with limited success.[63] Even the sensitive polymerase chain reaction does not provide definitive evidence for *M. paratuberculosis*–specific DNA in CD-affected bowel. Up to 65% of involved tissues may contain amplification products compared with 4% in patients with UC and 12% in controls,[64] and they can be found in granulomatous tissue. Collectively, these studies make only a marginal case for an important role of *M. paratuberculosis* in CD. The similarities between CD and Johne's disease in animals remain a puzzling and unresolved issue, but it is still plausible that *M. paratuberculosis* causes CD in a few patients.[65] Inconsistent results with antituberculous therapy have not helped to resolve these issues.[66]

Other transmissible agents. The possibility that a nonconventional bacteria or virus is related to CD has been considered after investigators induced chronic granulomatous inflammation in mice[67] or cytopathic effect in tissue cultures with CD tissue homogenates.[68] This line of investigation came to an end with the demonstration that the cytopathic effect of IBD tissue extracts was caused by toxic proteins.[69]

Measles Virus and Mesenteric Vasculitis

The complex challenges in defining "IBD-specific" agents are shown by studies on the measles virus. A vascular cause for the etiology of IBD was proposed more than a quarter of a century ago, but recently Wakefield et al. have provided more evidence for the involvement of the intestinal microcirculation in CD. Using resected specimens and an infusion technique, these investigators showed vascular injury, focal arteritis, fibrin deposition, and arterial occlusions in the inflamed segments.[70] In follow-up studies, they suggested that the vascular injury was caused by a diffuse granulomatous vasculitis.[71] Detailed examination of such intravascular granulomas showed the presence of paramyxovirus-like particles by transmission electron microscopy, compatible with the presence of the measles virus in CD tissues.[72] This suggestion was reinforced by positive hybridization for the measles virus N-protein genomic RNA and positive immunohistochemical staining for nucleocapsid protein in CD tissues, but only a minority of control intestinal tissues. Although the actual presence of measles virus in CD tissue has been challenged, the original group of investigators has provided epidemiological data indicating that perinatal exposure to measles virus increases significantly the risk of CD,[73] and measles vaccination increases the risk of developing IBD.[74] Finally, they showed that inherited disorders of coagulation protect against IBD.[75] Together, these observations could indicate a role for vascular abnormalities in CD pathogenesis, a hypothesis fitting with the increased risk of CD in people who smoke or use contraceptives. The notion of a vascular component in CD pathogenesis is credible, but whether it is specifically or exclusively caused by measles infection may be extremely difficult to prove.

Mediating IBD: Immune and Nonimmune Systems

Humoral Immunity

Mucosal B cells. When investigation switched from systemic immune events to those occurring in IBD-involved intestine, comparative mapping of B-cell distribution showed a massive increase in the number of plasma cells. This increase is not uniform with IgA, IgM, and IgG increasing 2-, 5-, and 30-fold, respectively.[76] This pronounced humoral immune response is particularly striking for IgG, the antibody class with the highest pathogenic potential. The two forms of IBD can be distinguished based on this Ig's subclasses: IgG1 increasing more in UC and IgG2 in CD. The reason for this difference is unclear, but distinct immune regulatory mechanisms or genetically conditioned differences may be responsible.[77] Abnormalities of J-chain expression are also present, both IgA2 and IgA1 being decreased in IBD.[78]

Autoantibodies and autoantigens. Much of the interest in studying humoral immunity in IBD is centered on whether disease-causing autoantibodies are produced. In a hallmark study in the late 1950s, Broberger and Perlmann described antibodies in the serum of UC patients that cross-reacted with human fetal colon cells in vitro.[79] The anticolon antibodies were not cytotoxic for colon cells, but this report was the first to highlight the concept of autoimmunity in IBD. Patients with IBD possess a variety of circulating antibodies that

seldom correlate with disease activity, e.g., cow's milk proteins.[80] Anticolon antibodies are not disease specific[81] and are not tissue specific, reacting, for instance, with biliary epithelium.[82] Antibodies to cytoskeletal proteins are frequent in IBD[83] but are also nonspecific, being found in many other disorders as well. Not all antibodies lack specificity. Serum antibodies to *Saccharomyces cerevisiae* (baker's yeast) are increased in patients with CD but not UC,[84] and a novel antierythrocyte autoantibody appears to have some specificity for CD.[85]

Validation for a role of autoimmunity requires the identification of autoantibodies that specifically recognize gut antigens and mediate tissue damage via this recognition. Candidate autoantigens have been isolated from intestinal epithelium (epithelial cell–associated components),[86] and sensitization to them has been shown in patients with IBD[87] and their relatives.[27] Still, none of them has been shown to cause gut inflammation. The best-defined autoantigen has been described by Takahashi and Das, who characterized a colonic protein of 40-kilodalton size exclusively recognized by IgG eluted from the colon affected by UC.[88] Monoclonal antibodies against this putative autoantigen identified a shared epitope in human colon, skin, and biliary epithelium as well as the eye and joints, locations compatible with the extraintestinal manifestations of UC.[89,90] This autoantigen appears to be the fraction 5 of the tropomyosin family of cytoskeletal proteins[91] and is codeposited with IgG1 and complement on UC colonocytes.[92] The true relevance of these findings for UC pathogenesis remains to be established.

Antineutrophil cytoplasmic antibodies. Antibodies against polymorphonuclear neutrophils (PMNs) or perinuclear antineutrophil cytoplasmic antibodies (pANCAs) have attracted the most interest.[93] A series of reports has confirmed the high prevalence (approximately 80%) of these antibodies in UC and an even higher prevalence in patients with primary sclerosing cholangitis.[94] A related finding is the increased incidence of pANCAs in healthy relatives of patients with UC and, to a lesser degree, patients with CD.[95] The prevalence of pANCAs in UC does not significantly vary in ethnically diverse populations,[96] but it does among unaffected relatives in different areas of the world.[97] In addition to being a potential marker of susceptibility and genetic heterogeneity in UC, pANCAs may define subsets of patients with IBD because recent studies suggest that pANCAs are more prevalent in patients with aggressive UC[98] and in those who develop chronic pouchitis after ileo-pouch anastomosis.[99]

A fundamental question is the true relevance of autoantibodies to UC in particular and IBD in general. pANCAs have yet to be proven to alter neutrophil function, although the recent demonstration of pANCA-producing B-cell clones in UC mucosa leaves the door open to alternate roles.[100] A related question is how many other autoantibodies exist in IBD and what is the range of their specificity. Humoral autoimmune phenomena are common in IBD, but their relevance to disease pathogenesis needs clarification, particularly because the simple occurrence of autoantibodies no longer indicates a pathological condition (horror autotoxicus), and it is part of normal immune homeostasis.

Cell-Mediated Immunity

Systemic immunity. Broberger and Perlmann were also the first to show the cytotoxic action of UC peripheral blood white blood cells against human fetal colon cells in vitro.[101] The cells mediating cytotoxicity were lymphocytes. These observations showed that immune cells could recognize and destroy intestinal cells, implicating classical immune mechanisms in IBD pathogenesis. Shorter et al. confirmed the action of blood lymphocytes against freshly isolated colonocytes[102] and eventually concluded that natural killer cells were responsible for this phenomenon.[103] These studies raised much excitement but relied on fairly crude assay systems, and the validity of the results is questionable.

Impaired natural killer cell activity has been described in IBD,[104] but other reports failed to confirm this observation. These early studies showed the inconsistency that still plagues immunologic data in IBD. Using mitogen-induced proliferation, some investigators detected impaired responsiveness in IBD,[105] a finding corroborated by anergic skin reactivity to dinitrochlorobenzene and depressed lymphocyte numbers in the circulation of patients with CD and UC.[106] In contrast, similar studies found no evidence for decreased immune reactivity in either subjects with CD or with UC.[107] Prolonged controversy also raged in trying to determine whether normal or defective generation of suppressor function is present in IBD, with different approaches providing conflicting results.[108,109]

Mucosal T cells. One important aspect of IBD, especially CD, is that the intestine can be infiltrated diffusely by immune cells in the absence of obvious morphological, clinical, and endoscopic evidence of inflammation. Aphthous ulcers are considered characteristic early lesions of CD, but how "early" such lesions really are in seemingly affected mucosa is debatable. Ultrastructural alterations,[110] increased numbers of $CD68^+$ macrophages,[111] and lymphocytic accumulation can go undetected in "unaffected" mucosa in patients with CD.[112] Thus, how and when mucosal immunocytes go awry is a

fundamental but unresolved question in IBD pathophysiology. In established lesions, the presence of T-cell infiltrates was detected by early immunohistochemical studies, but in contrast to other chronic inflammatory diseases, the proportions of $CD4^+$ and $CD8^+$ T cells were comparable in involved and uninvolved mucosa,[113] pointing to quantitative rather than qualitative changes in IBD tissue. Although the elevated numbers of T cells in the mucosa affected by either form of IBD suggest a connection between this cell type and tissue destruction, direct evidence is still lacking despite experimental data showing that activated T cells induce mucosal damage in organ culture.[114]

Mucosal T-cell function. Techniques allowing the isolation of immune cells directly from the mucosa constituted a major advance in the study of IBD. Although some differences were detected in the population of lamina propria compared with peripheral blood mononuclear cells, the proportions of T- and B-cell subsets were unexpectedly similar between IBD and control mucosa.[115] Not surprisingly, local cells in IBD showed an activated phenotype. Markers of lymphocyte activation, including the 4F2 antigen, the T9 transferrin receptor (CD71), and IL-2 receptor, are increased among isolated intestinal B cells and T cells and the $CD4^+$ and $CD8^+$ subsets,[116] as is the expression of IL-2, IL-2 receptor α, and β messenger RNA (mRNA).[117]

CD mucosal immunocytes show enhanced proliferation to gut-related and gut-unrelated bacterial antigens.[118] Increased, decreased, or normal suppressor cell activity by IBD lamina propria cells has been reported.[119,120] When mucosal mononuclear cells are activated with IL-2, striking differences are noticed not only between IBD and control but also between CD and UC cytotoxic activity. CD cells, despite producing low IL-2, show as much cytotoxicity as control cells, whereas UC cells that produce substantially less IL-2 show remarkably low killer activity.[121] These findings underscore the fact that, although T cells are activated in both CD and UC, this does not imply comparable behavior. In fact, preliminary evidence indicates that CD mucosal T cells are resistant to apoptosis, although UC T cells are not.[122]

The isolation of mucosal mononuclear cells also allowed direct studies of B-cell function in IBD. Studies by MacDermott et al. showed major alterations of Ig synthesis by IBD intestinal mononuclear cells, closely resembling those detected by immunohistochemistry, with an increased production of IgA, IgM, and particularly IgG, as well as altered patterns of monomeric IgA and IgA1.[123] The selected enhancement of IgG1 synthesis by UC and IgG2 by CD cells also agreed with immunohistochemical findings.[124]

T-cell receptor type and utilization. More recent studies have attempted to identify the distribution of the T-cell receptor in IBD, aiming at showing the preferential utilization of certain regions recognizing autoantigens, alloantigens, or superantigens as proposed for some autoimmune diseases.[125] Analysis of T-cell receptors in the peripheral blood showed clonal expansion of $CD4^+$ lymphocytes that persisted over time, suggesting a response to the same antigens in the course of the disease.[126] Another longitudinal study found no variation in the utilization of 22 Vβ genes in patients with CD, but a highly restricted Vβ repertoire was detected in distinct phases of the disease in those suffering from peripheral arthritis and ankylosing spondylitis.[127] An increased frequency of $\gamma\delta$ T cells has been described in the peripheral blood of patients with IBD and interpreted as the appearance of "gut-like" $\gamma\delta$ T cells in the circulation.[128] Immunohistochemical analysis, however, shows a relative decrease of $\gamma\delta$ T cells among intraepithelial and lamina propria lymphocytes in both CD and UC,[129] probably reflecting a relative increase in $\alpha\beta$ T-cell receptor cells in the inflamed gut.

Whether intestinal T cells are polyclonal or oligoclonal is also under active investigation. Initial studies indicated that lamina propria T lymphocytes were polyclonal.[130] Immunohistochemistry showed an increase in $V\beta8^+$ T-cell receptor cells in the mesenteric lymph nodes of some patients with CD,[131] but another study failed to identify a similar increase of $V\beta8^+$ cells.[132] The pattern of mucosal T-cell receptor Vβ expression apparently differs between circulating and mucosal cells in both patients with IBD and controls. In one study, the utilization of Vβ2, but not Vβ6, 7, and 14, was lower in patients with IBD, suggesting that T-cell receptor repertoire is altered in active IBD.[133] A subsequent study described a selected expansion of clonal T-cell receptor among $CD4^+$ lamina propria cells in the majority of CD isolates, and the investigators interpreted these finding as evidence of a response to a restricted set of disease-related antigens.[134] These observations are interesting but must be interpreted with caution, and whether receptor selectivity is linked to the pathogenesis of IBD disease or is simply secondary to chronic inflammation must be resolved. Skewed utilization of the T-cell receptor could indicate a response to neoantigens or superantigens but requires finding a restricted V region use in the majority of patients to implicate specific antigens in CD or UC pathogenesis. Although the number of intraepithelial T cells are not altered in either form of IBD, these cells can show enhanced expression of activation markers,[135] an altered $CD4^+/CD8^+$ cell ratio,[136] and alterations in their CD8-associated T-cell receptor usage.[137]

Monocytes and macrophages. Nonspecific cellular immunity is also altered in IBD. Both patients with CD and with UC overproduce monocytes, probably because of an increased demand of macrophages in the inflamed gut.[138] This explanation is supported by the finding in active IBD lesions of large numbers of recently recruited $CD68^+$, $L1^+$ blood monocytes.[139] Normal intestinal macrophages represent a heterogeneous cell population, and this heterogeneity is accentuated in IBD. The RFD1 antibody recognizing interdigitating antigen-presenting cells and the RFD7 antibody recognizing mature tissue macrophages detect abundant cells in IBD lesions, whereas clusters of $RFD9^+$ epithelioid cells are found predominantly in CD.[140] Macrophages are also evident in early IBD lesions, as shown by the presence of $HLA\text{-}DR^+$ and $ICAM\text{-}1^+$ cells in aphthous ulcers.[141] Thus, monocytic cells appear to be involved in all stages of IBD, underscoring their importance in IBD pathophysiology.

PMNs, eosinophils, basophils, and mast cells. Until recently the role of PMNs in IBD has received relatively little attention. The involvement of this cell in IBD pathogenesis was initially considered because of decreased migration rate in skin windows of patients with CD,[142] but this was thought to be a consequence of gut inflammation. This observation was followed by contradictory reports on PMN dysfunction in IBD, including decreased oxidative metabolism and superoxide dismutase level.[143] It is now clear that PMN products play a key role in the amplification of inflammation and tissue damage in IBD as discussed below. Reports on the number of tissue eosinophils and IgE-positive cells in IBD lesions are contradictory. Some studies show an increase of these cells in the mucosa, but this is not a consistent finding, and the presence of elevated numbers of eosinophils and IgE-positive cells is probably secondary to inflammation. There is also evidence of mast cell activation and degranulation in IBD. Enhanced histamine levels are common in the mucosa and intestinal secretions of patients with UC and with CD, but this is also likely to be a secondary, nonspecific event associated with inflammation.[144]

Nonimmune Cells

All intestinal cells are affected in active IBD. Early transmission electron microscopy studies showed damage to vascular epithelium and autonomic nervous fibers, as well as myofibroblastic transformation of smooth muscle cells.[145] These studies provided indirect evidence for the physical and functional involvement of nonimmune cells in IBD pathogenesis, an area that is now attracting considerable attention.[146]

Epithelial cells. One of the most important developments in intestinal immunity is the realization that local immune cells are functionally integrated with cells of nonimmune origin. Among these, epithelial cells have been studied the most extensively, and their reciprocal interaction with the adjacent immunocytes is well recognized. Human enterocytes and colonocytes can express class II antigens and can function as antigen-presenting cells,[147] playing a role in inflammation. Evidence of epithelial cell involvement in IBD derives from studies showing that they express functional cytokine receptors for the common γ chain of the IL-2 receptor; respond to IL-15; secrete IL-7, which activates lamina propria mononuclear cells; produce chemokines, colony-stimulating factor, and TNF-α; and express leukocyte adhesion molecules.[148–151] Thus, it is not surprising that abnormalities of epithelial cell phenotype and function are found in IBD. Immunohistochemical studies show diffuse expression of histocompatibility antigen by colonic epithelium in both CD and UC,[152] and epithelial cells express activation antigen such as the 4F2 antigen, transferrin receptor, and acute lymphoblastic leukemia antigen.[153] A potentially important abnormality of colonic epithelium in IBD is a defective capacity to induce suppressor T cells. Although normal epithelial cells preferentially activate $CD8^+$ suppressor T cells, a function that might contribute to induction of local tolerance, IBD epithelial cells preferentially activate $CD4^+$ helper T cells, perhaps leading to an amplification of local immune reactivity and inflammation.[154] This exciting possibility awaits confirmation.

Mesenchymal cells. A variety of other nonimmune cells actively participate in IBD pathogenesis, including "structural" cells such as fibroblasts, myofibroblasts, and muscle cells. Human intestinal fibroblasts differ from skin fibroblasts. They produce a variety of extracellular matrix proteins, but this production is altered in IBD, as exemplified by the increased production of collagen type III in strictured areas of CD.[155] Muscularis propria cells also produce increased amounts of collagen in IBD.[156] These alterations are associated with classical pathological features of IBD, such as the increased proliferation of muscle cell layers, formation of thickened bowel wall, and strictures. The function of mesenchymal cells in normal and IBD mucosa is almost certainly broader than previously suspected. Preliminary reports show that intestinal fibroblasts also express multiple cell surface molecule and activation markers, modulate leukocyte adhesion, bind immune cells, and prolong T-cell survival.[157,158]

Nerve cells. A still unresolved question is whether stressful events of life initiate, trigger, or somehow

influence IBD. Although epidemiological evidence relating life stress to IBD symptoms is scant, there is documentation that stress modulates inflammatory responses.[159] A link between the neuroendocrine and immune systems in the gut can only occur via the enteric nervous system, and the immunomodulation of enteric neuromuscular function has important implications for both motility and inflammation.[160] Qualitative abnormalities of nerve fibers in IBD tissue have been long recognized,[161] and quantitative changes have been described in both CD and UC, although they appear secondary to inflammation.[162] Nevertheless, there is also evidence of changes related to a specific type of inflammation, such as the different concentration and receptor density expression for substance P in CD- vs. UC-involved mucosa.[163,164] The beneficial effect of topical anesthetics reported in ulcerative proctitis is provocative but requires additional investigation.[165]

Endothelial cells. Another important nonimmune cell that regulates mucosal homeostasis is the microvascular endothelial cell. These cells serve as "gatekeepers" of inflammation because of their critical position in relation to the process of extravasation of circulating leukocyte into the interstitium. Patients with IBD show indirect evidence of endothelial damage and a prothrombotic state, but these are almost certainly secondary to intestinal injury.[166] A far more important question is whether the gut endothelium contributes directly to IBD pathogenesis. Recently developed techniques allow the isolation and study of this highly specialized cell type, and initial observations indicate that the mucosal endothelium possesses unique functional features.[167] These features are altered in IBD because intestinal mucosal endothelial cells show a markedly enhanced capacity to bind leukocytes compared with cells from normal mucosa.[168] Enhanced binding by IBD endothelial cells persists regardless of in vitro culture time, suggesting that persistence of inflammation may result, at least in part, from a hyperadhesive intestinal microvasculature. Similar functional modifications may very well occur for other cell types, ultimately creating a self-perpetuating proinflammatory loop sustained by the activity of nonimmune cells, a mechanism underlying other types of chronic inflammation.[169]

Mediators of Immunoregulation and Inflammation

Immunoregulatory cytokines. The prototypic immunoregulatory cytokine is IL-2 and this molecule has been evaluated extensively in IBD. Circulating IL-2 is generally not detectable in health or disease, but decreased IL-2 bioactivity by isolated human intestinal mucosal mononuclear cells was first reported in both CD and UC.[170] A subsequent study using reverse-transcription polymerase chain reaction found increased IL-2 mRNA in active CD but not UC lesions,[171] a discrepancy likely caused by diverse assay systems. More importantly, CD and UC T cells differ markedly in their response to IL-2. When activated by this cytokine, UC mucosal T cells respond weakly, in contrast to CD cells that show a hyperreactive response.[121] This hyperreactivity complements other evidence, suggesting that IL-2 is involved intimately in CD pathogenesis: CD mucosal T cells show the highest expression of IL-2Rα gene products,[117] administration of IL-2 to patients with CD causes clinical exacerbations,[172] and patients with CD who develop acquired immunodeficiency syndrome and lose IL-2–secreting T cells enter clinical remission.[173]

The investigation of cytokine receptors has provided complementary insights into mechanisms of IBD. Healthy individuals have circulating levels of soluble IL-2 receptor α, and these increase in a variety of pathological conditions, including IBD in which levels correlate with clinical activity.[174] This correlation must be interpreted with caution because peripheral soluble IL-2 receptor α levels may reflect mucosal inflammatory activity in CD, but systemic immunity in UC[175] and the source of increased mucosal expression of IL-2 receptor α are T cells in CD but macrophages in UC.[176]

The production of interferon gamma (IFN-γ) by intestinal mucosal mononuclear cells was also initially reported to be decreased in both forms of IBD.[177] Subsequent reports suggested particular relevance of this cytokine to CD, as indicated by the spontaneous release of IFN-γ and increased IFN-γ mRNA expression by lamina propria mononuclear cells and the presence of IFN-γ–secreting T cells in actively inflamed mucosa.[178,179] Induction of IFN-γ is strictly dependent on IL-12, a monocyte/macrophage-derived cytokine consisting of two separate subunits (p35 and p40 chains). Enhanced spontaneous and mitogen-induced production of both IL-12 subunits by CD, but not UC or normal, mucosal cells and tissues has just been reported recently.[180] This observation also strengthens the suggestion of a so-called Th-1–like pattern in CD and helps differentiate the immunopathogenesis of CD and UC.

Information on other immunoregulatory cytokines in IBD is limited and inconsistent. Production of IL-4 by both CD and UC mucosal immune cell cultures has been reported as decreased,[181,182] whereas elevated IL-4 mRNA in UC but not CD mucosal biopsy specimens was found in another study.[183] These results should be interpreted in the context of a recent study showing that early postsurgical recurrence of ileal CD is associated with significantly

elevated IL-4 mRNA levels, suggesting distinct patterns in acute vs. chronic inflammation.[184] Of interest is that the down-regulatory effect of IL-4 on activated circulating mononuclear cells is attenuated in IBD,[185] a finding compatible with an impaired systemic and mucosal anti-inflammatory activity in IBD.

Information about IL-5 is no less confusing. Recurrent CD is associated with eosinophilic infiltration and high IL-5 mRNA levels by in situ hybridization.[186] However, IL-5 protein production by cultured mucosal cells is seemingly decreased in CD but increased in UC,[182] whereas another study finds elevated IL-5 mRNA in UC but not CD biopsy specimens.[183] Both patients with CD and with UC with active disease have increased circulating levels of IL-10,[187] and this cytokine might act as an anti-inflammatory agent in IBD mucosa.[188]

Proinflammatory cytokines. In contrast to immunoregulatory cytokines, proinflammatory cytokines tend to be consistently elevated in IBD. Serum levels of IL-1 are seldom detected even in patients with severe disease, but high concentrations of IL-1 are found in both CD and UC intestine,[189] largely attributable to local mononuclear cells.[190] The biological effects of IL-1 are determined in part by the relative content of IL-1 receptor antagonist, a natural antagonist that occupies the IL-1 receptor without triggering inflammation. There is evidence for a mucosal imbalance between IL-1 receptor antagonist and IL-1 in IBD, with a relative deficiency of IL-1 receptor antagonist levels caused by excessive synthesis of IL-1.[191] Increased production of IL-1 is not specific for IBD because it is found in other forms of gut inflammation,[192] and a similar imbalance is found in other chronic inflammatory conditions. Of particular relevance is a reported association between the allele 2 of the IL-1 receptor antagonist gene and UC. Carriage of at least one copy of this allele increases the chance for developing UC or a more severe form of this disease, but not in CD.[39]

Although most proinflammatory cytokines tend to be elevated consistently in IBD mucosa, serum, protein, and mRNA content of TNF-α have been variable. A British group found elevated TNF-α concentrations in sera of children with active UC and colonic CD and in stools of children with both types of IBD.[193,194] In contrast, another study failed to detect differences in TNF-α serum levels between children with and without IBD.[195] Production of TNF-α is greater in cultures of CD than UC mucosal mononuclear cells,[196] and direct evaluation of IBD tissue sections by in situ hybridization shows elevated TNF-α mRNA in macrophages,[197] but other reports found no differences in TNF-α mRNA expression between control and IBD mucosal biopsy specimens.[198] TNF-α has been the target of clinical investigations aimed at blocking its activity as a novel form of therapy for CD, but the mechanism of the apparent beneficial effects of a chimeric monoclonal anti–TNF-α antibody is unclear.[199]

Circulating levels of IL-6, a broad spectrum cytokine with characteristics of an acute-phase reactant, are peculiarly high in patients with active CD but not in patients with UC.[200] In contrast, IL-6 is elevated consistently in IBD tissues, where its source is primarily macrophages and epithelial cells.[201] Chemokines, or chemoattractant cytokines, mobilize and attract PMNs (through IL-8) and monocytes (through monocyte chemoattractant protein 1). Serum levels of IL-8 are a poor marker of IBD clinical activity, even though tissue homogenates contain extremely high concentrations of this chemokine in both UC and CD.[202] The source of IL-8 in the mucosa is still incompletely defined because of the possible contribution of epithelial cells to its production. Some studies using in situ hybridization to localize the cellular origin of IL-8 in active IBD lesions identified macrophages, neutrophils, and epithelial cells, but other studies failed to find IL-8 mRNA expression in the intestinal epithelium.[203,204] Increased monocyte chemoattractant protein 1 is present in both biopsy specimens and intestinal epithelial cells from patients with active IBD.[205]

The study of cytokines has been the most active area of IBD investigation. Still, consensus about the key soluble mediators of CD and UC escapes us. This is in part because of an ever-expanding number of identified cytokines, their multiplicity of action, and cellular sources. In addition, the above-mentioned discrepancies have created considerable difficulty in the interpretation of individual results, particularly if one attempts to create a global picture of the immunoregulatory status of patients with IBD. These discrepancies may reflect the use of different methodological approaches and arbitrary sample selection, major pitfalls in cytokine assessment. For instance, increased production of IL-1β, TNF-α, and IL-6 is observed even in microscopically normal CD mucosa,[206] and cytokine profiles change during clinical evolution,[184] casting doubts about results based on the selection of "normal" and inflamed tissue by subjective criteria or lack of detailed attention to clinical parameters of disease. The indiscriminate comparison of whole biopsy specimens vs. purified cells, stimulated vs. unstimulated cultures, short-term vs. long-term cultures, and bioactive protein vs. immunoreactive protein vs. mRNA complicate interpretation of results and our understanding of immunoregulation in IBD. Nevertheless, these studies have already generated a whole new spectrum of therapeutic possibilities that may have a lasting impact on the way we approach treatment of IBD.

Mediators of Healing and Injury

Growth factors. Growth factors are a class of soluble mediators increasingly recognized as having a role in IBD pathogenesis. From in vitro animal and human studies, there is strong indication for a crucial function of growth factors in prevention of mucosal injury, and healing after injury has occurred. Transforming growth factor (TGF)-β is a dominant mediator of intestinal epithelial restitution and defense.[207] Trefoil peptide, which is produced in large amounts by epithelial cells in IBD,[208] has a unique molecular structure conferring protection against inflammatory damage.[209] Increased expression of keratinocyte growth factor, a stromal cell-derived mitogen specific for epithelial cells, is also observed in areas actively involved by IBD,[210] implicating mesenchymal cells in the modulation of epithelial cell renewal during inflammation. The exact role of each growth factor in IBD is still being defined, but clearly, individual factors have distinct functions in different phases of IBD. In active CD and UC lesions, TGF-α is produced in quantities comparable to those present in normal mucosa, but TGF-β production is increased.[211] In contrast, when IBD activity enters in a quiescent phase, TGF-α levels increase, whereas TGF-β levels normalize. Thus, enhanced TGF-β synthesis may represent an effort to promote healing, but augmented TGF-α production may cause epithelial hyperproliferation and increase the risk of malignancy in IBD.

Eicosanoids. Eicosanoid is a term used to describe multiple products with both proinflammatory or anti-inflammatory activity that derive from the breakdown of membrane phospholipids into arachidonic acid and subsequent formation of bioactive substances, which include prostaglandins (PGs), thromboxanes, and leukotrienes.[212] A link between eicosanoids and IBD was shown initially after discovering that the mucosa of patients with UC contained high levels of PGs whose synthesis was inhibited by the use of sulfasalazine.[213] Other eicosanoids were subsequently found to be elevated in both UC and CD, including thromboxanes, prostacyclines, and leukotriene B_4.[214,215] Although uniformly high during inflammation compared with those present in normal mucosa, profiles of eicosanoid are distinct in each type of inflammation. Concentrations of PGE_2, thromboxane B_2, and leukotriene B_4 are markedly elevated in UC compared with those of CD and *Clostridium difficile*–induced colitis.[216]

Animal models of colitis have suggested an important contribution of eicosanoids to IBD, and manipulation of arachidonic acid metabolism has therapeutic implications for gastrointestinal inflammation. Although inhibition of eicosanoids by nonsteroidal anti-inflammatory drugs is beneficial in some animal models, this is not the case in humans, in whom these drugs trigger clinical exacerbations of IBD.[217] The specific inhibition of selected eicosanoids is under intense investigation, and much emphasis currently has been placed on cyclooxygenase (COX)-1 and 2, constitutive and inducible enzymes, respectively, critical for PG production. Whether PGs can improve IBD because of cytoprotective effect or are harmful through their proinflammatory activity is a key issue. Efforts to develop selective inhibitors such as COX-2 inhibitors that preserve cytoprotection while inhibiting inflammation are justified, but their ultimate effect in humans remains to be determined. Preliminary evidence suggests that COX-2 inhibitors with antipeptic ulcer activity may be detrimental in IBD because experimental colitis is exacerbated by treatment with an anti–COX-2 drug.[218] Unrelated to eicosanoids, another mediator of IBD is platelet-activating factor (PAF). Mucosal production of this substance is elevated in UC,[219] but this is a nonspecific event because elevated PAF levels are found in other forms of intestinal inflammation.

Reactive oxygen and nitrogen metabolites. Abundant infiltration by PMN has long been considered a hallmark of active IBD, but the role of these cells in inflammation and tissue injury has not been investigated adequately. The contribution of PMNs to IBD pathogenesis has come under renewed attention after recognition that they are the main source of potent toxic molecules such as reactive oxygen metabolites (ROMs) and reactive nitrogen metabolites. With the explosion of interest in nitric oxide as a crucial signaling and bioactive molecule in the gastrointestinal tract, its role in IBD has become the focus of a new investigation. There is evidence from animal models that ROMs are involved in gut inflammation,[220] and the same appears to be true for NO through the modulation of inducible NO synthase.[221] Inhibition of inducible NO synthase by compounds such as L-arginine analogues can significantly decrease the extent and severity of tissue injury in experimental colitis.[222]

These findings may have a parallel in human IBD. Chemiluminescence probes show large quantities of ROMs in the mucosa of patients with CD and with UC that correlate with disease severity.[223] Elevated NO synthase activity was reported initially in UC but not CD colonic tissue, but subsequent studies showed that NO generation and synthase activity are increased in both forms of IBD.[224,225] Normal colonic epithelium does not express inducible NO synthase activity, but its expression is induced in the epithelium involved by IBD and other types of inflammation.[226] The functional role of NO in IBD is far from settled in view of its dual toxic and

protective effects. A complementary aspect to the elevation of ROMs and NO in inflammation is the observation that IBD mucosa is relatively depleted of antioxidant defenses, rendering it susceptible to oxidative injury,[227] and reactive metabolites cause direct epithelial cell damage in active IBD.[228] This has led to the assumption that limiting production of these highly reactive molecules may improve IBD. Adding methylprednisolone to mucosal organ culture decreases NO activity,[225] and sulfasalazine, mesalamine, and olsalazine have a scavenger effect on superoxide radical formation.[229] This indirectly suggests that some of the therapeutic effect of drugs commonly used in IBD are mediated by their antiradical activity.

Short-chain fatty acids. Among many hypotheses for the pathogenesis of IBD, there is the intriguing suggestion that the colonic epithelium in UC fails to adequately oxidize butyrate, perhaps resulting in an energy-deficient condition of the mucosa.[230] Although this hypothesis has raised limited interest, it has not been abandoned and was recently reinforced by intriguing clinical observations that irrigation of diversion colitis with short-chain fatty acids results in marked improvement[231] and that butyrate enemas may induce clinical improvement in distal UC.[232]

It is obvious that cytokines and other mediators are all produced and act concomitantly in the inflamed gut. This creates an extremely complex mixture of bioactive molecules influencing each other while at the same time mediating tissue injury and repair. The aggregate outcome determines persistence vs. resolution of IBD, and the present challenge is to understand which molecules should be rightfully chosen as ideal therapeutic targets.

Mediators of Cell Contact

Cell adhesion molecules. Adhesion molecules represent a large family of molecules essential for cell communication, activation, and homing. In view of the gut infiltration by inflammatory cells, a fundamental role of these adhesion molecules is expected in IBD. Recruitment of leukocytes into the mucosa occurs during active disease,[233] a process quantitatively and qualitatively abnormal because leukocyte homing patterns in IBD are disrupted because of loss of selectivity of intestinal lymphocytes binding to mucosal and peripheral lymph node vessels.[234] Aberrant expression of cell adhesion molecules in areas of inflammation is confirmed by several reports. In active IBD, mucosal mononuclear phagocytes show a dramatic increase in expression of ICAM-1.[235] In addition, marked increase of ICAM-1 and leukocyte function antigen 1 expression by mononuclear cells and E-selectin by venules is also found.[236,237] Surprisingly, the expression of vascular cell adhesion molecule 1 by immune and endothelial cells is not increased in IBD when compared with control mucosa. Although these changes could be nonspecific consequences of inflammation, there is also evidence that an aberrant display of adhesion molecules differs in the two major forms of IBD. For instance, there is an increased expression of CD44 v6 and CD44 v3 in UC but not CD colonic crypt cells.[238] Additionally, CD, UC, and normal intestinal T and B cells show variable integrin patterns, perhaps representing different homing properties in each type of bowel inflammation.[239] To some degree, the dysregulation of adhesion molecule expression in the gut is reflected in the peripheral circulation, where circulating levels of soluble adhesion molecules, such as ICAM-1, are found during active disease.[240]

A logical assumption originating from the above findings is that the blocking adhesion molecule expression may stop recruitment of inflammatory cells in the gut, inhibiting or eliminating the intense cell-cell communication that contributes to chronic immune activation. Several animal models of IBD have been used to assess the effect of cell adhesion molecule antibodies with encouraging results. Administration of $\alpha 4$ monoclonal antibody attenuates colitis in the cotton top tamarin,[241] and antibodies against CD11b/CD18 or ICAM-1 improve colitis induced by various exogenous agents.[242,243] Similarly, antibodies against molecules critical to lymphocyte homing are effective in down-regulating inflammation. Antibodies to the gut homing integrin $\alpha 4\ \beta 7$ led to a rapid resolution of chronic colitis in the cotton top tamarin.[244] The ultimate goal of these novel approaches is to achieve beneficial effects by manipulating adhesion molecule expression in patients with IBD. Preliminary results observed in steroid-dependent patients with CD being treated with ICAM-1 antisense oligonucleotides promise a potential beneficial effect in this situation.[245]

Mimicking IBD: Animal Models

In view of the experimental limitations imposed by studies of human disease, an animal model reproducing many, if not all, of the IBD characteristics would be extremely valuable. Early attempts to study experimental gut inflammation met with limited success. Spontaneous colonic lesions reported in the rat, mouse, gibbon, swine, and dog were sporadic, and the models were impractical. Allergic or hypersensitivity reactions induced colitis, as well as radiation, vitamin deficiency, lymphatic obstruction, rectal or systemic administration of proteolytic enzymes, stress, and cholinergic drugs, but all have dubious relevance to IBD.[246] An ideal animal model must

enable study of crucial questions, such as what microbes cause IBD, what genes underlie disease expression, how many mechanisms are involved, what distinguishes primary from secondary phenomena, and whether specific agents can be tested for therapeutic value. Most of these issues can now be addressed because of the progress achieved during the last decade, which has yielded excellent animal models of IBD.[247,248]

Animal Models and IBD Pathogenesis

Spontaneous models of IBD are rare, and only the cotton-top tamarin has received serious consideration as a model of UC. Experimental colitis can be induced easily by a variety of chemical or natural substances, the most common being acetic acid, dextran sodium sulfate, trinitrobenzene sulfonic acid, peptidoglycan polysaccharide, indomethacin, and carrageenan. Immune system reconstitution can also induce gut inflammation, as with the transfer of CD45RB^{high} T cells into mice with severe combined immunodeficiency disease or bone marrow cells into cyclosporine-treated mice. Mutant animals have been generated using molecular genetic techniques as either transgenic, e.g., with a dominant or dominant-negative expression of a specific gene product, or "knockout," e.g., with the targeted deletion of a specific gene product. The list of transgenic and knockout animals that develop intestinal inflammation continues to expand (Table 3). The appearance of gut inflammation could be anticipated in some models, as for chemically induced injury and IL-10–deficient mice, and perhaps predictable in others such as the HLA-B27/β2m transgenic rats, but it was unexpected in the IL-2, IL-2 receptor α, or major histocompatibility complex class II knockout mice or totally surprising, as for the $G\alpha_{i2}$-deficient or the CD45RB^{high}-reconstituted mice with severe combined immunodeficiency disease.

Some aspects of the biology of IBD animal models deserve special mention because of pathogenic and thera-

Table 3. Bowel Inflammation in Knockout or Transgenic Animals

Targeted gene or gene product	Animal	Type of defect	Pathology	Microflora influence	Immunologic abnormalities	Genetic background effect
IL-2	Mouse	Knockout	Acute and chronic colitis in entire colon (worst distal)	Required	Cytokine imbalance, not B cell mediated, $CD4^+$ T cells involved	Likely
IL-2 receptor α	Mouse	Knockout	Anemia; colitis	Unknown	Lymphoproliferation, lack of cell death?	Likely
IL-10	Mouse	Knockout	Enterocolitis	Required	Increased TH1, $CD4^+$ T cells involved	Unknown
TGF-β1	Mouse	Knockout	Multiorgan inflammation, mild gastritis and colitis	Unknown	Lack of control of T-cell activation; MHC class I and II increased; some cell adhesion molecules increased	Highly dependent
$G\alpha_{i2}$	Mouse	Knockout	Colitis, distal to proximal; adenocarcinoma	Unknown	Abnormal thymic development; IgG2α and IgM increased; mature T cells increased	Yes
N-cadherin	Mouse	Transgenic chimera	Focal enteritis, neoplasms	Unknown	Crypt epithelium disruption	Unknown
TCR-α^- or β^- TCR-$\beta^- \times \delta^-$	Mouse	Knockout	Colitis, milder for TCR-$\beta^- \times \delta^-$	Unknown	Unrestrained humoral response (B cells)? NK cells involved?	Yes
MHC class II	Mouse	Knockout	Acute colitis, distal to proximal	Unknown	B cells involved?	Yes
HLA-B27 and β_2m	Rat	Transgenic	Enterocolitis, gastritis, carditis, epididymitis, arthritis	Required	Increased IFN-γ, $CD4^-$ cells involved	Yes
CD3ε26	Mouse	Transgenic	Colitis after marrow transplantation	Unknown	Lack of thymic selection; increased IFN-γ, $CD4^+$ cells involved	Unknown
CD45RB-reconstituted SCID	Mouse	Reconstitution of immunodeficient mouse with purified lymphoid populations	Enterocolitis	Required	Mediated by CD45RB^{hi}, $CD4^+$ T cells, increased IFN-γ, increased TNF-α	Unknown

Modified from Morales et al.[248]

MHC, major histocompatibility complex; NK, natural killer; SCID, severe combined immunodeficiency disease; TCR, T-cell receptor.

peutic implications for human disease. A fundamental aspect is that microbial agents are apparently needed to develop colitis in several models. This is exemplified by a rat model in which injection of bacterial cell wall components causes chronic granulomatous inflammation with intestinal and extraintestinal manifestations.[249] Compelling evidence for the importance of enteric bacteria in IBD comes from HLA-B27 transgenic rats. These animals develop inflammation in multiple organs, but small and large bowel are spared when rats are kept in a germ-free environment.[250] Similar results are observed in IL-2– and IL-10–deficient mice. The reverse is also true, e.g., reconstitution of germfree HLA-B27 transgenic rats with normal luminal bacteria, especially *Bacteroides* species, reinstates gut inflammation.[251] Whether and which bacteria or combination of bacteria are relevant to IBD in humans remains to be determined.

Continuous antigenic challenge leads not only to gut inflammation but also to a defect of epithelial barrier function that, in turn, may contribute to chronicity of inflammation.[252] In some models, sensitization to specific antigens can induce inflammation, a phenomenon with therapeutic implications because the oral administration of the hapten trinitrobenzene sulfonic acid causes immune unresponsiveness (tolerance) in animals in which colitis has been induced by this hapten.[253] If specific agents cause human IBD through an antigen-specific sensitization process, then the oral intake of the antigens could theoretically induce tolerance and resolution of gut inflammation.

The emerging concept that intestinal inflammation is not exclusively caused by an excess of inflammatory damage but also by a relative lack of anti-inflammatory activity is strengthened by animal models. In acute colitis of rabbits induced by immune complexes, Cominelli et al. have shown that expression of IL-1 gene products is a key early event for the induction of inflammation and that specific blockade of the IL-1 receptor down-regulates inflammation.[254] In this model, administration of recombinant IL-1 receptor antagonist blocks inflammation, whereas neutralization of IL-1 receptor antagonist exacerbates and prolongs colitis.[255]

The pivotal importance of T cells in developing experimental IBD is supported by several lines of evidence. Breeding experiments with IL-2–deficient mice show that the animals still develop colitis when crossed with B-cell ($JH^{-/-}$)-deficient mice but not when crossed with mice lacking T and B cells ($RAG2^{-/-}$).[256] Although T cells appear to be indispensable to many forms of experimental colitis, whether this is because of T-cell abnormalities later in life or congenital defects is still an open question. Supporting the second possibility is the appearance of IBD in Tgϵ26 mice with aberrant thymic selection after reconstitution with T cells.[257] Abnormalities of T-cell function may not need to be a primary developmental defect because modification of the intestinal immune system may result in IBD. Early (1-month) appendectomy in the T-cell receptor α–mutant mice prevents the subsequent development of colitis, recapitulating the protective effect of appendectomy reported in humans for the risk of UC.[258,259]

Other models suggest that the etiology of IBD is not related to immune abnormalities but rather to a defect of intestinal barrier function, alone or associated with other triggers. Mice deficient in keratin 8 and those dominant/negative for *N*-cadherin develop colitis as well as adenomas or carcinomas.[260,261] Partial defects of mucosal protective mechanisms also result in inflammation. Mice lacking intestinal trefoil factor are normal until a mild epithelial injury occurs, after which extensive colitis ensues.[262] Thus, it is reasonable to assume that both a single or combined defects, either of primary or secondary nature, may underline the pathogenesis of chronic intestinal inflammation.

Table 4. Agents With Reported Therapeutic Efficacy in Animal Models of IBD

Soluble mediators	Mediator blockers	Adhesion molecule inhibitors	Drugs	Dietary products	Miscellaneous agents
IL-4, IL-10, IL-11	Cytokine inhibitors	α_4-Integrin antibody	Corticosteroids	Fish oil	Antibiotics
Fibroblast growth factor	Cytokine receptors	LFA-1 antibody	Aminosalicylates	Eicosapentanoic acid	Anti-CD4 antibody
Keratinocyte growth factor	Growth factor receptors	$\alpha_4\beta_7$ antibody	Misoprostol	Butyrate	Antioxidants
Calcitonin gene-related peptide	Eicosanoid inhibitors	ICAM-1 antibody	16-16 Dimethyl PGE_2	Plant extracts	Hyperbaric oxygen
	Eicosanoid receptor antagonists	ICAM-1 antisense	Ketotifen		Heparin
	Neuropeptide antagonists		Verapamil		Factor XIII
	NO inhibitors		Bosentan		Nicotine
	PAF receptor antagonists		Bismuth		Glutamine
			Zinc sulfate		Cysteine
			Benzalkonium chloride		Methionine
			Rebamipide		Ursodeoxycholic acid
			Genistein		NF-κB antisense

LFA-1, lymphocyte function–associated antigen 1.

Table 5. Outstanding Questions in IBD Pathogenesis

Disease presentation	Is CD a disease or a syndrome?
	Do other forms of IBD exist in addition to CD and UC?
Environmental factors	What environmental changes favor the appearance of IBD?
	Why does smoking affect UC and CD in totally opposite ways?
Familial and genetic factors	Is increased intestinal permeability in healthy relatives of patients with CD a primary or secondary phenomenon?
	How much genetic heterogeneity exists among patients with IBD?
	What clinically useful genetic markers exist for CD and UC?
Microbial agents	Does any specific microorganism cause UC or CD?
	Is the whole or selected enteric flora involved in IBD pathogenesis?
Immune and nonimmune cells	Which cell type is central to gut inflammation?
	Are the same cells responsible for acute and chronic IBD?
Immune response	Do immune abnormalities specific for CD and UC exist?
	Can CD and UC be distinguished based on diverse immune reactivity?
	Is any form of IBD a true autoimmune disorder?
Cytokines and mediators	Do regulatory cytokines develop well-polarized patterns in CD or UC?
	Does any specific inflammatory mediator directly cause tissue damage?
Tissue damage	Is any specific cell type targeted by the mucosal immune response?
	Is tissue damage completely nonspecific?
Primary vs. secondary events	Are primary etiopathogenic events still present in chronic IBD?
	Do late secondary events perpetuate gut inflammation?
Therapy	Will specific mediator-targeted therapy be more effective than broad immunosuppression?

In cell-transfer models of colitis, selected T-cell subsets have gut inflammation-inducing capacity, such as the $CD45RB^{high}$-positive T cells, whereas $CD45RB^{low}$ fail to induce colitis or have a protective effect.[263] This model is characterized by production of high levels of IFN-γ and TNF-α, and antibodies against IFN-γ and TNF-α prevent and limit the development of colitis, respectively.[264] The disparate efficacy of the neutralizing antibodies suggests that elevation of cytokine content in IBD does not always predict the pathogenic role of a mediator.

Poorly understood but often-mentioned factors in association with the development and course of IBD are stress and environmental agents. The involvement of stress has been explored in cotton-top tamarins that develop spontaneous colitis only when kept in long-term captivity.[265] The appearance of chronic colitis in these primates has been reported to correlate with stressful conditions.[266]

Animal Models, Human IBD, and IBD Therapy

The clinical and pathological features of intestinal inflammation differ in each animal model, but some characteristics suggest direct relevance to and even commonalities with human IBD: (1) clinical manifestations clearly depend on the genetic background; (2) alterations of multiple and unrelated immunoregulatory molecules can lead to inflammation with similar characteristics; (3) T cells, but not B cells, are needed to develop inflammation; (4) intestinal inflammation is not solely dependent on an excessive immune activation but also on the balance between proinflammatory and anti-inflammatory responses; and (5) several models fail to develop experimental colitis in a germfree environment. These general conclusions strongly support the current assumption that different and independent abnormalities cause IBD and that UC and CD are heterogeneous disorders with multiple etiologic and pathogenic mechanisms.

In addition to offering new insights into the pathogenesis of intestinal inflammation, animal models offer an opportunity to test new therapeutic agents. Although experience has shown that several agents are beneficial in the treatment of experimental IBD, drugs of totally different origin, composition, and function appear to be effective in animal models with completely diverse mechanisms of inflammation (Table 4). Although the enormous list of "effective" agents points to a lack of specificity of inflammatory pathways, the efficacy of each therapy can make sense depending on the model, particularly in those with a well-defined immunologic mechanism. In the mouse model of trinitrobenzene sulfonic acid–induced colitis, modulation of the cytokines predicted to be important for sustaining immune reactivity is effective: administration of antibodies against IL-12 abrogates even established inflammation, as does induction of oral tolerance resulting in TGF-β–mediated resolution of colitis.[267,268] The possibility that mechanisms responsible for IBD in animals and humans are diverse is real, but whether imbalances of immunoregulatory, proinflammatory, and immunosuppressive cytokines can be manipulated in humans as effectively as in animal models remains to be proven.

Reasoning IBD: Realities and Expectations

At the end of an intricate fiction book the reader expects to know "who did it," but at the end of a review

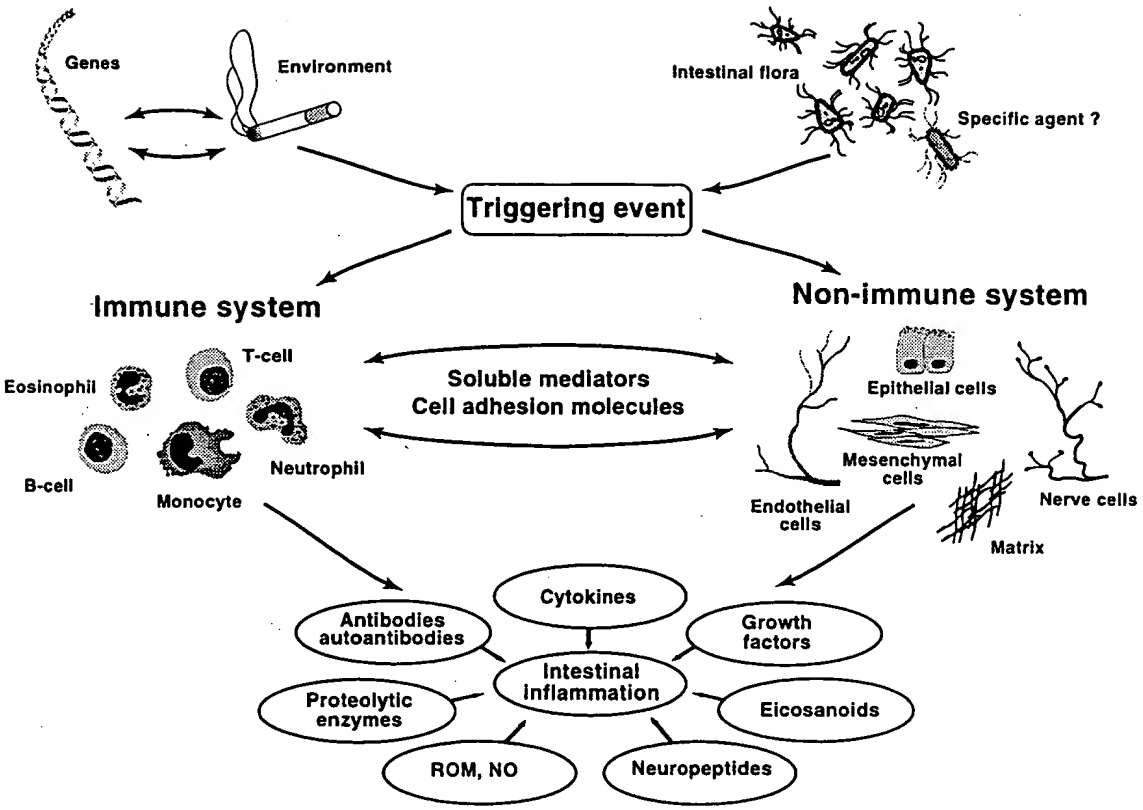

Figure 1. Diagram showing components and events involved in IBD etiopathogenesis. Interacting environmental and genetic factors in combination with the microbial intestinal flora or a still unidentified specific microorganism trigger an event that activates intestinal immune and nonimmune systems. The cell-mediated immune response induces effector T cells and activates macrophages, neutrophils, and other leukocytes, whereas the humoral response stimulates B cells to produce antibodies. Through secretion of soluble mediators and expression of cell adhesion molecules, immune and nonimmune cells exchange signals, resulting in further cell activation and amplification of the production of antibodies and autoantibodies, cytokines, growth factors, eicosanoids, neuropeptides, ROMs, NO, and proteolytic enzymes culminating in inflammation and tissue damage.

on the etiopathogenesis of IBD, the reader will not know "who did it." The story on IBD is still unfolding, and outstanding questions prevent the final chapter from being written (Table 5). There is no lack of strong clues or circumstantial evidence, but how exactly the protagonists of the plot, e.g., environmental, genetic, microbial, cellular, and molecular factors, conspire to make IBD happen is still not in our grasp, but it is close (Figure 1).

A great deal of the difficulty in understanding IBD is caused by two unique features of the gut: (1) a huge antigenic load from dietary and bacterial products in the lumen and (2) the presence of "physiological inflammation." In the healthy intestine, these elements are controlled by powerful mechanisms, but when IBD ensues, they turn into confounding factors that camouflage what could otherwise be an explicit inflammatory response. On the background of a myriad of microorganisms, identifying a single microbial agent responsible for CD or UC may be impossible. Despite this difficulty, there is a growing interest in gut ecology. Whether the enteric flora should be targeted as a whole vs. selected groups of microorganisms or whether the search for a "*H. pylori* equivalent" should be intensified are open questions. Similarly, on the background of a complex and dynamic mucosal immune system, the identification of immune derangements also requires special efforts.

Not all factors relevant to IBD pathogenesis are masked by the features of the intestine because environmental and genetic factors come into play before IBD is manifested. It is unlikely that either one can cause IBD on its own, but different mixtures of environmental and genetic factors may result in a similar IBD phenotype. This latter scenario is increasingly attractive as the number of IBD "genes" is expanding, perhaps explaining the wide spectrum of clinical manifestations in CD and UC. A legitimate concern is to end up with too many genes linked to IBD, in which case the genetic background would be relatively less important to the development of the disease, whereas the contribution of environmental factors would become more important. If this were the case, a concerned effort should be made to study IBD in its very early stage, perhaps in pediatric populations where masking of primary pathogenic events may not have occurred.[269]

The ultimate goal of understanding IBD is to provide relief to the patients by controlling those factors that went awry. We have no means of altering the genes or the environment, but we have learned a great deal about inflammatory mechanisms in the gut. As a consequence, biological revolution to IBD therapy is under way,[270] combined with a sensible management of the gut ecology.[271] Unfortunately, the results of such rationale and scientific approach are still short of expectations. There is overwhelming evidence that inappropriately activated T cells play an important role, but drugs that cause T-cell down-regulation can be of limited value.[272] Targeting potent proinflammatory mediators can be extremely effective in vitro or in animals but may fail in patients.[273] Perhaps abnormalities we detect in the laboratory are secondary events of which IBD is independent, or we may be treating IBD too cautiously or too late, when irreversible changes have occurred. Modern science and technology have given us two gifts: an unprecedented level of sophistication to study inflammation inside and outside of the gut and a bounty of new products for clinical trials. If we do not lose sight of the natural history of the disease and the complexity of its clinical manifestations, these gifts will bear the fruits of a full understanding of the cause and mechanisms of IBD.

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Received August 8, 1997. Accepted February 10, 1998.

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Supported by grants DK30399 and DK50984 from the National Institutes of Health and by the Crohn's & Colitis Foundation of America, Inc.

The author thanks Dr. Jeffry A. Katz for critical review of the manuscript.

Acta med. Croatica, 51 (1997) 37–40

ANIMAL MODELS OF INFLAMMATORY BOWEL DISEASE

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The aim of this article was to reveal the major biologic features of intestine that predispose the intestinal mucosa to numerous inflammatory conditions, especially in regard to experimental models of inflammatory bowel disease. The need for the greater understanding of the etiology of intestinal inflammation and the search for more effective and novel therapy for the treatment of the disease has led to the development of variety of experimental models of inflammatory bowel disease. There is a growing number of animal models of inflammatory bowel disease, either naturally occurring in several mammalian species or inducible in various species of experimental animals by using physical, chemical and biologic agents including the embrionic stem cell technology for specific gene targeted defects. Despite some serious objectives in regard to clinical aspects of human intestinal bowel disease, animal models of intestinal inflammation have advantages and being complementary to clinical approach indicate the clear need for experimental studies to be continued.

Key words: animal models, inflammatory bowel disease

Received for publication: April 20, 1995

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Diseases of the digestive system account for the occupancy of great number of hospital beds, maybe more than any other group of disorders. There is a growing body of evidence that the inflammation, including immune reaction, participate in the pathogenesis of a wide range of digestive diseases.[1, 2]

The major biologic features of the intestine are transmembrane absorption and secretion involving the complexity of both local and distant neural and endocrine factors that contribute to the regulation of intestinal motility, also maintaining the barrier between the host and potentially harmful pathogens and mutagens.[1, 2] This is mainly accomplished through both physical integrity of the intact mucosal surface and extensive population of resident immune cells.[1–3] The presence of luminal noxious agents, together with the potential disruption of mucosal surface and concentration of so many immune cells capable of activating the cascade of inflammation, predisposes the intestinal mucosa to numerous inflammatory conditions.[3] However, the normal intestinal immune system is under a careful balance in which proinflammatory and antiinflammatory cells and molecules are usually regulated in order to promote a normal host mucosal defense capability without destruction of intestinal tissue.[1]

The intestinal inflammation is grossly characterized by macroscopic occurrence of edema, ulceration and hemorrhage, and necrosis, which is consistent with histologic evidence of accumulation of inflammatory cells and release of inflammatory mediators such as cytokines, leukotrienes, chemotactic substance and reactive oxygen species. However, the results of extensive experimental studies suggest that, in spite of the wide nature of physical, chemical and biologic agents involved in the pathogenesis of intestinal inflammation, the final pattern of macroscopic, pathohistologic and biochemical parameters of inflammation remains relatively uniform.[1, 3–17]

Ulcerative colitis and Crohn's disease remain diseases with both acute and chronic inflammatory features of unknown etiology. Several hypotheses have been proposed to explain the cause and nature of the observed intestinal and extraintestinal changes in these diseases. According to most studies, serious consideration is given to the infective agents and immunologically mediated injury.[11, 12, 17] Recent studies have documented the importance of luminal exposure to potent, nonspecific stimulatory bacterial products which are capable to evoke the state of activation of the intestinal immune system, resulting in marked up–regulation of mucosal inflammatory pathways.[1]

The need of the greater understanding of the etiology of intestinal inflammation and search for more effective and novel therapy for the treatment of the disease have led to the development of a variety of experimental models. There is a growing number of animal models of inflammatory bowel disease (IBD) induced in several mammalian species by various chemical and biologic agents.

The ideal model for investigations should be either naturally occurring or inducible in mammalian species, and identical to all clinical aspects of the human disease.[4, 5] This means that the animal disease is induced and maintained by the same primary and secondary factors (has the same etiology and pathophysiology), has an equivalent clinical

spectrum, and is treatable with the same therapeutic agents.[4–7] Additionally, the ideal model must be a practical study tool (easy accessibility, easy experimental manipulation and not expensive).

CATEGORIES OF ANIMAL MODELS

There are two main categories of animal models of IBD: one in which intestinal inflammation occurs naturally in various mammalian species, and the other in which the dissease is experimentally induced, either by exposure to dietary substances, chemical/pharmacological agents or materials derived from patients or by manipulation of the animal's immune system.[4–7]

Naturally occurring intestinal inflammation

Two interesting models of spontaneous acute and chronic colitis were found, documented and characterized in cotton–top tamarin (primate species *Saguinus oedipus*)[18] and juvenile rhesus macaques.[19] These models are featured by the development of active colitis that is not associated with identifiable pathogens and, as in humans, the activity of the disease process spontaneously vaxes and wanes.

Experimentally induced colitis

This broad category of experimentally induced colitis differs greatly according to the noxious agents or events involved.

1) Intestinal inflammation in mice, rats, hamsters, guinea–pigs or rabbits induced by oral administration or rectal instillation of physical or pharmacological agents (acetic acid, sulfated polysaccharides such as carrageenan, amylopectine sulfate, dextran sulfate or chemotactic peptides and free radical initiators[8–10, 13–16]

2) Attempts were made to induce intestinal inflammation by injecting homogenized and filtrated Crohn's disease tissue into mice or rats, but with equivocal effects as documented in different studies.[11, 12]

3) Inflammatory injury of intestinal mucosa induced by manipulation of animal's immune system. a) B–cell models in rabbits, in which the intestinal inflammation is induced by the mechanisms of cross–reacting antibodies and immune complexes, mainly using preimmunization or injection of preformed immune complexes with subsequent topical irritation of colonic mucosa with diluted formalin;[20,21] b) T–cell models that involve activation of lymphocytes by sensitization with haptens and subsequent rectal challenge of the cell–mediated immune system in mice, rats, guinea–pigs and rabbits;[8, 10, 16] c) ulcerative colitis–like disorder in interleukin–2 or interleukin–10 deficient mice, using embryonic stem cell technology for specific gene targeted defects.[22, 23] In these genetically engineered animal strains that are likely to develop intestinal inflammation, when raised in a totally germ–free (gnotobiotic) environment, intestinal inflammation does not develop. This demonstrates the central importance of bacterial pathogens or bacterial cell wall products or toxins in these particular animal model.[1] The investigation of these new gene–targeted murine inflammatory disorders is an exciting prospect, although their exact relevance to the human condition is uncertain.

The authors of this review have also contributed to the study of a wide variety of T–cell models by developing a simple and reproducible model of intestinal inflammation, defined by the use of 2,4–dinitrofluorobenzene (DNFB) challenge enema in previously sensitized mice.[10, 24, 25] Local application of 2,4–DNFB into the colon of previously sensitized mice resulted in the acute and chronic colonic injury. Stimulation of the local immune system was observed throughout the murine intestine. Moreover, extraintestinal lesions such as pericholangitis and lymphofollicular proliferation in the spleen were observed.[25] The role of several drugs that modify immune response as well as the role of free oxygen radical scavengers are currently under investigation in this model. Some of the pathohistologic features of this DNFB induced experimental colonic injury are discussed in the paper published in this issue of *Acta Medica Croatica*.[29]

In regard to clinical aspects of human IBD, animal models of intestinal inflammation have major advantages as well as serious objectives. These animal models enable *in vivo* studies under the controlled laboratory conditions, and with well defined genetic impact they offer the possibility of changing/modulating the entire course of the provoked disease. Furthermore, it is possible to obtain tissue specimens from adequate anatomical structures at any time in the course of the disease. On the other hand, the models on small animals inhibit the study of disease progress in an individual animal due to inconvenience of endoscopic surveillance. Although some models are reported to develop extraintestinal manifestations of the disease, this is unusual in such induced models and they do not necessarily develop the intraintestinal complications of colon cancer.[4] Thus, experimentally induced models of intestinal inflammation do have some limitations. However, most of the currently used animal models involve the induction of acute and subacute inflammatory lesions, and are possibly best suited for the study of inflammatory mediators, potential therapeutic strategies[26] and the healing process.[4, 6]

* * *

A survey of the abstracts submitted at the 10th World Congress of Gastroenterology in Los Angelse, USA in 1994, showed that animal models of experimental colitis were used in more than 30 studies.[27] However, at the 1st Croatian Congress of Gastroenterology in Zagreb (1994), none of the submitted abstracts dealt with the methodology of experimentally induced intestinal inflammation.[28]

Many different animal model studies were undertaken in the past decades in order to study inflammatory disease in colon. This approach to the

problem of IBD is complementary to the clinical approach. It makes possible to analyze different steps in the pathogenesis of injury separately. Moreover, despite many similarities with human inflammatory bowel disease, the differences cannot be ignored. It is important to emphasize that even in the presence of such differences according to species or possible mechanisms studied, there is a clear need of experimental studies to be continued. Furthermore, human IBD although remaining the disease of unknown etiology and pathogenesis, urges the search for more effective therapy thus making this option obligatory.

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S A Ž E T A K

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Interna klinika Kliničke bolnice »Dubrava«, Zavod za kliničku imunologiju i reumatologiju[1] i Zavod za gastroenterologiju[2] Interne klinike Kliničkog bolničkog centra u Zagrebu

Mnogi biološki čimbenici čine sluznicu crijeva sklonom nastanku upale, što je temelj za razvoj modela crijevne upale u eksperimentalnih životinja. Eksperimentalne studije upalne bolesti crijeva potaknute su potrebom za boljim razumijevanjem etiologije i patogeneze crijevne upale, kao i potrebom za razvojem novih i učinkovitijih terapijskih postupaka u svrhu liječenja upalne bolesti crijeva u ljudi. Brojni modeli upalne bolesti crijeva opisani su kao prirodno nastala upalna bolest crijeva u nekih vrsta sisavaca ili kao eksperimentalna crijevna upala u pokusnih životinja izazvana primjenom fizikalnih, kemijskih i bioloških činitelja, uključujući i postupke genetskog inženjeringa. Unatoč određenim razlikama u odnosu na kliničku sliku upalne bolesti crijeva u ljudi, eksperimentalni modeli crijevne upale u pokusnih životinja imaju bitne prednosti te njihova znanstvena utemeljenost predstavlja komplementaran pristup kliničkim studijama upalne bolesti crijeva u ljudi.

Preliminary Report on the Mount Sinai Hospital Inflammatory Bowel Disease Genetics Project

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BACKGROUND: Although the etiology of inflammatory bowel disease (IBD) is unknown, there is increasing evidence that genetic predisposition plays a major etiologic role. To provide the framework for gene identification using a positional cloning approach, ascertainment of families with multiple affected members and careful documentation of pedigrees are essential. Objective: To report the initial findings of the IBD Genetics Project of the Mount Sinai Hospital IBD Research Unit. METHODS: All records of patients with ulcerative colitis and Crohn's disease followed at the Mount Sinai Hospital IBD Unit were reviewed. A questionnaire was sent to all patients to ascertain those with a family history of IBD. Patients with a presumed family history were contacted by a research assistant, and after confirmation of diagnosis, relevant clinical information, pedigrees, and consent to contact family members were obtained. Blood for DNA and cell line preparation were collected from affected and nonaffected family members. RESULTS: Of 2,504 patients registered in the IBD database, 231 (9.2 percent) were found to have an affected family member: 96 of 964 (10 percent) with Crohn's disease (CD) and 135 of 1,540 (8.8 percent) with ulcerative colitis (UC). A mean of 2.4 family members were affected. In families in which the proband had CD, 82.3 percent had only two affected family members, 78.1 percent had only family members affected with CD, and 82.3 percent had only first-degree family members affected. In families in which the proband had UC, 70.4 percent had only two affected family members, 71.1 percent had only family members affected with UC, and 65.2 percent had only first-degree family members affected. In the 231 families, there were 103 sibling pairs: 46 percent with CD, 28 percent with UC, and 26 percent with CD/UC. CONCLUSION: These data suggest that approximately 10 percent of IBD patients have affected family members, with the rate being similar in UC and CD. Future research is directed to genome scanning and linkage analysis in this cohort of patients. [Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Genetics; Family history]

McLeod RS, Steinhart AH, Siminovitch KA, Greenberg GR, Bull SB, Blair JE, Cruz CR, Barton PM, Cohen Z. Preliminary report on the Mount Sinai Hospital Inflammatory Bowel Disease Genetics Project. Dis Colon Rectum 1997;40:553-557.

Although there have been advances in the understanding of the underlying pathophysiologic mechanisms of inflammatory bowel disease, the fundamental cause(s) remains unknown. Although a number of factors have been suggested as playing etiologic roles, there is strong evidence to suggest that there is a genetic predisposition for its development.[1] This includes epidemiologic evidence that inflammatory bowel disease (IBD) is more common in certain countries and ethnic groups, most notably Ashkenazi Jews.[2-5] Furthermore, approximately 10 to 20 percent of patients with ulcerative colitis (UC) or Crohn's disease (CD) report a family history of IBD, and the risk to family members of affected individuals has been estimated to be between 10 and 30 times higher than the risk to the general population.[6-8] Although these data indicate a key role for genetic factors in development of IBD, the epidemiologic observations also indicate that inheritance of IBD is complex and not likely to follow a simple Mendelian mode.

With availability of polymorphic microsatellite markers, covering the entire genome, and with semi-automated technology, rapid and complete genome scanning is now feasible, thus paving the way for isolation of the specific genes responsible for the spectrum of disorders. For example, the genes responsible for cystic fibrosis, amyotrophic lateral sclerosis, and Huntington's chorea have recently been identified using genetic linkage and other positional cloning techniques. With these advances in mind, the Mount Sinai Hospital Inflammatory Bowel Disease Genetics Project was initiated in 1994.

This project represents a collaborative effort that

Read at the meeting of The American Society of Colon and Rectal Surgeons, Seattle, Washington, June 9 to 14, 1996, and the Tripartite Meeting, London, United Kingdom, July 8 to 10, 1996.
No reprints are available

includes surgeons, gastroenterologists, molecular geneticists, genetic biostatisticians, research nurses, and data management personnel. Although the ultimate goal of the project is identification of the genetic abnormalities responsible for Crohn's disease and ulcerative colitis, initial efforts have concentrated on the ascertainment of families with multiple affected individuals, development of family pedigrees, and collection of blood samples for DNA cell line preparation. Thus, the objective of this report is to report the initial findings in the MSH patient population.

MATERIALS AND METHODS

Patient Identification

Initially, the names of patients with a family history of IBD were recalled by physicians, surgeons, and nurses. Subsequently, a systematic approach to identifying all patients with inflammatory bowel disease who were registered with the Mount Sinai Hospital IBD Unit was undertaken. The office charts of all physicians and surgeons with a specific interest in treatment of IBD were reviewed. Names of these patients were cross-referenced with those contained in a database of information on all patients who had undergone a pelvic pouch procedure and selected patients who had medical or surgical treatment for Crohn's disease at this institution since 1984. In doing so, it was felt that all patients with a diagnosis of ulcerative colitis and Crohn's disease were identified.

Methods

A questionnaire was mailed to all identified patients with IBD to ascertain whether they had other affected family members. Patients in whom a family history was elucidated from office records or the returned questionnaire were contacted by a research nurse. A family pedigree was then developed using the Cyrillic® (Cherwell Scientific, Oxford, United Kingdom) software program, and clinical and/or pathologic evidence to confirm the diagnosis of IBD and to determine whether the patient had ulcerative colitis or Crohn's disease was obtained. For patients who were not followed at our institution, pathologic slides were obtained for review, or relevant clinical information was reviewed by one of the senior investigators. Other clinical information recorded was age at onset, sites and pattern of disease, presence of extraintestinal manifestations, and surgical history. Demographic data and information on possible risk factors such as ethnicity, smoking, blood transfusions, and measles vaccination history were also recorded. All data were recorded on a database using SIR software (SIR Pty. Ltd., Milsons Point, New South Wales, Australia).

Consent was also obtained so other affected and nonaffected family members could be contacted by telephone or mail. Because affected sibling pair analysis does not require knowledge of the mode of inheritance in polygenic/multifactorial diseases, initial efforts were concentrated on families with two or more affected siblings.[9] Blood samples were obtained from all affected family members plus the mother and father of affected siblings; DNA was extracted, and cell lines were established whenever possible. The study was approved by the University of Toronto Review Committee on the use of Human Subjects.

RESULTS

Two thousand five hundred four patients with IBD registered in the Mount Sinai Hospital IBD Unit were identified. Of these, 964 (38.5 percent) have been diagnosed as having Crohn's disease and 1,540 (61.5 percent) as having ulcerative colitis. Mailed questionnaires were sent to all of these patients, of whom 79 percent responded. To date, 231 (9.2 percent) patients (probands) with at least one affected family member have been identified. Of these, 135 (8.8 percent) have ulcerative colitis, and 96 (10.0 percent) have Crohn's disease. In addition to the proband, 312 (mean, 1.4) affected family members have been recruited into the study. Further information regarding these families is listed in Table 1.

Of the 231 families with multiple affected members, 103 (44.6 percent) include 2 or more affected siblings. Family pedigrees of 57 of these families have been

Table 1.
Affected Relatives of Crohn's Disease and Ulcerative Colitis Probands

Affected Relatives	Crohn's Disease Proband (%)	Ulcerative Colitis Proband (%)
First degree	79 (82)	88 (65)
Second degree	7 (7)	30 (22)
Both	10 (10)	17 (13)
Crohn's disease only	75 (78)	31 (23)
Ulcerative colitis only	15 (16)	96 (71)
Both	6 (6)	8 (6)
% With one affected relative	61 (64)	95 (70)
% With two affected relatives	28 (29)	30 (22)
% With >2 affected relatives	7 (7)	10 (7)

completed. Forty-six percent of these families have members affected with CD only, 28 percent with UC only, and 26 percent with both CD and UC (Fig. 1). Mean number of affected siblings per family is 2.1.

DISCUSSION

Two approaches may be taken in an attempt to determine the genetic abnormality/abnormalities that confer susceptibility to a genetic disorder.[1] The first is to identify candidate genes (*i.e.*, genes that have already been cloned and that are known to encode proteins that are potentially relevant to development of the disease) and to determine whether these genes differ in any way in patients compared with unaffected individuals. For instance, in IBD, it has been postulated that genes located within the human leukocyte antigen (HLA) complex on chromosome 6 may contribute to disease susceptibility. Although the candidate gene approach has certain merits, the disadvantage is that the chance of choosing the correct susceptibility genes from among the 50 to 100,000 genes present in the human genome is unlikely and, thus, a great deal of effort may be expended with little or no yield. The second approach used for gene identification in complex genetic diseases is positional cloning, a strategy predicated on the localization of the disease gene(s) using polymorphic markers and linkage analysis. Although positional cloning represents a very useful approach to identification of the genes underlying human disease, this strategy requires several sequential steps (Table 2). First, families with multiple affected individuals must be identified to permit the search for markers that cosegregate with the disease. In this regard, analysis of affected sibling pairs (*i.e.*, 2 or more affected siblings) is a particularly powerful approach, because this analysis allows one to search for disease susceptibility genes in situations in which the inheritance pattern and penetrance are unknown. No matter what types of families are collected, care must be taken to ensure that the diagnosis and other clinical information are correct in both affected and nonaffected members. Accurate pedigrees must also be developed and blood samples collected from all study participants. Polymorphic microsatellite markers are used to scan the entire human genome, followed by nonparametric statistical analysis to look for linkages between the disease and microsatellite marker loci. Finally, when significant linkages are identified, physical mapping techniques are used to isolate the specific genes responsible for the disease. Once the genes are identified, genotype/phenotype correlations can be made to better understand the biologic abnormalities, to identify at risk patients, to classify further patients with regard to screening, and finally, to direct therapy and to develop new and more specific therapies.

In this study, susceptibility genes for IBD will be sought using the positional cloning approach. For diseases such as IBD in which the etiology likely involves interaction between several genes, it is probable that some individuals in a family will carry some of the predisposing genes but not manifest the disease. In addition, there may be variable gene penetrance, etiologic heterogeneity, variable age of onset, and presence of phenocopies (*i.e.*, nongenetic cases)

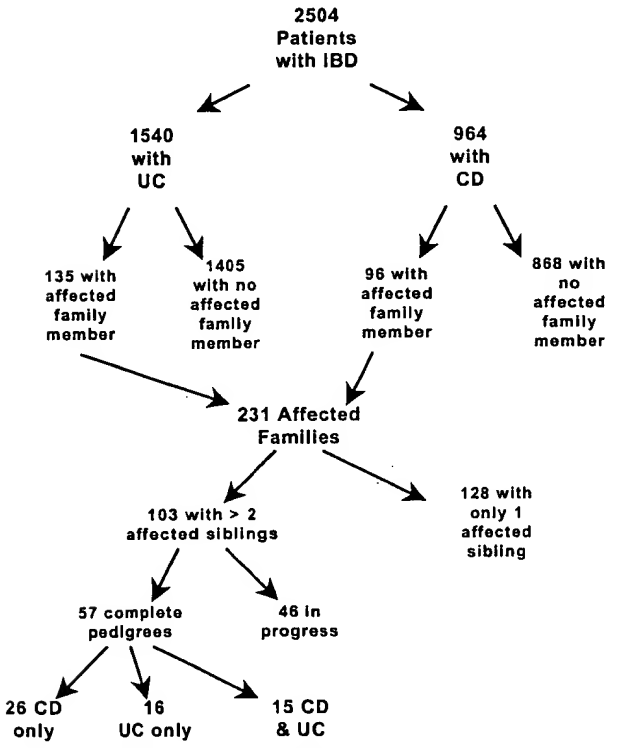

Figure 1. Distribution of patients registered at the Mount Sinai Hospital Inflammatory Bowel Disease (IBD) Unit according to disease and family history. UC = ulcerative colitis; CD = Crohn's disease.

Table 2.
Steps in the Identification of Genetic Abnormalities

1.	Ascertainment of affected families and development of pedigrees
2.	Collection of DNA
3.	Genome scanning and linkage analysis
4.	Physical mapping
5.	DNA sequencing
6.	Gene characterization

that interfere with linkage assessment in polygenic diseases. For these reasons, it is estimated that 100 to 200 families with 2 or more affected siblings may be necessary to provide statistically significant linkage data. For the same reasons, a similar-sized set of families will be used to replicate linkage findings. Although many patients may have a family history of IBD, not all will have affected sibling pairs, and blood samples may not be available from both affected and nonaffected nuclear family members. Thus, to ascertain an adequate number of DNA samples from sibling pair families, a large number of families must be identified and their family pedigrees documented.

To date, we have been successful in identifying a large cohort of families with multiple affected members. Our preliminary results suggest that approximately 10 percent of patients with inflammatory bowel disease have a family history. The proportion is similar among both Crohn's and ulcerative colitis patients. In the literature, between 12 and 35 percent of patients have been reported to have a family history. This discrepancy may reflect differences in defining the inception cohort and referral biases but may also reflect variability in disease prevalence in different populations.[1, 8, 10–13] Although other reports have suggested that patients with CD are more likely to have a family history than those with ulcerative colitis, we detected no difference in the frequency of family history among UC and CD patients.

We have concentrated our initial efforts on ascertaining Crohn's disease as opposed to ulcerative colitis families because epidemiologic data suggest that the heritability of CD is greater than that of UC. For example, results of twin studies indicate disease concordance in monozygotic twins to be approximately 50 percent in Crohn's disease but much less for ulcerative colitis.[14] Importantly, as disease concordance is not 100 percent for either CD or UC monozygotic twins, these data also indicate an important role for nongenetic factors in development of these diseases.

Previous studies of IBD etiology have demonstrated an apparent association between UC and HLA-DR2 and between Crohn's disease and HLA-DR4.[15–19] Although data from such studies have demonstrated some degree of genetic heterogeneity between UC and CD, to date they have yet to provide substantial insight into the underlying disease-promoting genetic factors. In addition, some studies of HLA associations in IBD have not demonstrated a significant HLA-disease linkage in multicase CD families. Recently, Satsangi *et al.*[20] performed linkage analysis on 74 families in which 2 or more siblings had IBD and in which included a total of 83 sibling pairs. Among this group, 42 sibling pairs had Crohn's disease, 29 had UC, and 12 had one sibling with CD and one with UC. In addition, these investigators performed an association study in which 175 patients had UC and 173 had CD, and there were 472 controls. An analysis of the sharing of alleles among affected sibling pairs indicated linkage of UC with the DR-B1 locus, but no HLA gene linkage with CD. In addition, no overall association of HLA alleles with CD was observed. Although these data do not preclude a role for HLA genes in IBD causation, they suggest that the contribution is small and possibly only relevant to a limited subgroup of patients. These data together with the strong evidence from concordance rates in twin pairs that genetic factors are important in the pathogenesis of CD suggest that for IBD the important disease susceptibility genes lie outside the HLA region.

There are two recently published reports of the results of genome scanning in IBD families.[21–22] By using a nonparametric sibling pair linkage method to study 25 affected sibling pairs with 270 highly polymorphic markers, Hugo *et al.*[21] identified a putative CD susceptibility locus on chromosome 16. These findings were subsequently replicated in another panel of 53 families that included one or more generations in the analysis. More recently, Satsangi and coworkers[22] reported strong linkages of IBD with loci on chromosomes 3, 7, and 12. Their data were obtained using 89 sibling pair families and were subsequently replicated by analysis of 97 sibling pairs. Linkages identified involved three microsatellite markers on chromosome 3, four on chromosome 7, and three on chromosome 12.

Our long-term efforts are also aimed at linkage analysis and physical mapping. Currently, in addition to the 103 sibling pairs identified in the MSH patient population, another 107 have been identified from other sources. Genome scanning has been initiated, and data analysis is currently pending. Because of the careful documentation of clinical information, this study should provide an opportunity to not only identify IBD genes but also examine the association of particular genes with different disease patterns and manifestations.

CONCLUSIONS

Approximately 10 percent of IBD patients in our population have affected family members. The pro-

portion of UC and CD patients with a family history is similar. Although the majority of affected families have members with the same disease, approximately 25 percent of families include both Crohn's disease and ulcerative colitis affected members. Furthermore, more than 30 percent of families have three or more affected persons. Future studies will concentrate on genome scanning, linkage analysis, and ultimately physical mapping and gene isolation. However, identification of patients with a family history, development of complete pedigrees, and collection of accurate clinical data are essential prerequisites to the positional cloning of genes for UC and CD.

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SYMPOSIUM: BIOSECURITY AND DISEASE

Johne's Disease: A Hidden Threat

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ABSTRACT

Paratuberculosis, which is also known as Johne's disease, is a chronic, progressive enteric disease of ruminants caused by infection with *Mycobacterium paratuberculosis*. Cattle become infected with *M. paratuberculosis* as calves but often do not develop clinical signs until 2 to 5 yr of age. The clinical disease is characterized by chronic or intermittent diarrhea, emaciation, and death. Although animals with clinical disease are often culled from the herd, animals with subclinical paratuberculosis may cause economic losses because of reduced milk production and poor reproductive performance. Although the economic impact of paratuberculosis on the national cattle industry has not been determined, it is estimated to exceed $1.5 billion/yr. The diagnosis of subclinical paratuberculosis is difficult. Bacteriologic culture is the most definitive method of diagnosis, but culture is time consuming and labor intensive. Serological assays are not very useful because animals do not develop an antibody response until the clinical stages of disease. Development of assays to measure cell-mediated immunity is critical to accurate detection of paratuberculosis in subclinically infected animals. Although not considered a zoonotic agent, *M. paratuberculosis* has been identified in intestinal biopsy tissue from patients with Crohn's disease, an inflammatory enteritis in humans. Currently, the potential human health risk is being addressed by research evaluating pasteurization of dairy products in the US.

(**Key words**: Johne's disease, ruminants, control)

Abbreviation key: **AGID** = agar gel immunodiffusion, **CF** = complement fixation, **IFN** = interferon, **PCR** = polymerase chain reaction.

INTRODUCTION

The disorder known as paratuberculosis was first described in 1895 by Johne and Frothingham (23) who identified organisms in granulomatous lesions in the intestines of affected cattle that stained acid-fast, indicating some type of mycobacterial organism. The organism was cultured from cattle in 1910 and was classified as a mycobacterium by Twort (50) and Twort and Ingram (51). The organism was fully characterized several years later and named *Mycobacterium paratuberculosis*. Paratuberculosis is widely distributed both nationally and internationally in domesticated ruminants such as cattle, sheep, goats, as well as deer, antelope, and bison. The prevalence of the disease in the US is difficult to ascertain because fully comprehensive studies have not been conducted to date. Based upon culture of *M. paratuberculosis* from the ileocecal lymph nodes of culled cattle at slaughter, a study published in 1987 (30) reported that the national prevalence of bovine paratuberculosis in both dairy and beef cattle approached 1.6%. However, major dairy states, such as Pennsylvania, Wisconsin, and California have reported estimates of 7.2, 10.8, and 3.1% infection in culled dairy cows, respectively (1, 5, 57). A Wisconsin study (12) reported an estimated 34% herd infection rate based upon serological diagnosis of paratuberculosis in dairy herds throughout the state. The accuracy of those estimates is limited by the sensitivity of the diagnostic tests used (culture or serologic), accurate recognition and reporting of the disease, and number of animals sampled. It is estimated that losses in the US from paratuberculosis in cattle herds may exceed $1.5 billion/yr (25). This figure is extrapolated from estimated values of prevalence, computation of financial losses from culling or death of clinically infected cows, and reduced reproductive efficiency, feed efficiency, and decreased milk production in subclinically infected animals. The significance of subclinical infection on economic losses to the producer are detailed in a recent review (24); a reduction in milk production of 15 to 16% accounts for the major portion of net monetary loss (1, 4). Cows that are infected with paratuberculosis beyond second lactation have demonstrated losses of 1300 to 2800 lb (590 to 1270 kg) of milk per lactation (59).

Received March 31, 1997.
Accepted September 30, 1997.

DISCUSSION

Manifestation of Disease

Cattle become infected with *M. paratuberculosis* as calves but often do not develop clinical signs until 2 to 5 yr of age (29). The primary route of infection is through ingestion of fecal material, milk, or colostrum containing *M. paratuberculosis* microorganisms. Once ingested, *M. paratuberculosis* survive and replicate within macrophages in the wall of the intestine and in regional lymph nodes. After an incubation period of several years, extensive granulomatous inflammation occurs in the terminal small intestine, leading to malabsorption and protein-losing enteropathy. Cattle shed minimal amounts of *M. paratuberculosis* in their feces during the subclinical phase of infection, and yet, over time, this shedding can lead to significant contamination of the environment and an insidious spread of infection throughout the herd. During the clinical phase of infection, fecal shedding of the microorganism is quite high and can exceed 10^{10} organisms/g of feces (8). The terminal clinical stage of disease is characterized by chronic diarrhea, rapid weight loss, diffuse edema, decreased milk production, and infertility. Although transmission of paratuberculosis occurs primarily through the fecal-oral route, *M. paratuberculosis* has also been isolated from reproductive organs of infected males and females. Viable organisms have been isolated from fetuses of infected cows, although intrauterine transmission of *M. paratuberculosis* has not been proven (8).

Diagnosis of paratuberculosis is difficult because of the fastidious growth pattern of the microorganism and because of the paradoxical immune response of the host animal to infection. During the early subclinical stages of infection, the microorganism elicits a cell-mediated response by the host that can be characterized by strong delayed-type IV hypersensitivity reactions, lymphocyte proliferative responses to mitogens, and production of cytokines by stimulated T lymphocytes. As the disease progresses from subclinical to clinical stages, the cell-mediated immune response wanes, and a strong humoral response predominates. The presence of antibody to *M. paratuberculosis* does not protect the host against the disease; indeed, active cell-mediated immunity appears to be essential to keep the infection in check. During the final stages of disease, lack of antigen-specific cell-mediated immune response or complete anergy may result, allowing for rapid dissemination of the infection throughout the host (3).

Diagnosis of Paratuberculosis

Bacteriologic culture is the most definitive method of diagnosis, but culture is time consuming, requiring up to 12 wk of incubation, and also labor intensive (9). Contamination is often a problem when *M. paratuberculosis* is being cultured from fecal specimens, and the National Animal Disease Center has recently incorporated a two-step decontamination procedure to reduce the amount of fungal and bacterial microorganisms significantly (43). Because animals with subclinical disease may shed organisms intermittently in their feces, use of fecal culture alone as a diagnostic method may result in misrepresentation of infection in the herd; only about 50% of *M. paratuberculosis* is detected by fecal culture (39).

Serologic tests for diagnosis of paratuberculosis such as agar gel immunodiffusion (**AGID**), ELISA, and complement fixation (**CF**) are relatively easy to perform but are not sensitive (10, 28, 40). The AGID is a simple procedure based upon antigen-antibody precipitation in agar and is most often used as a rapid, diagnostic method for confirmation of clinical paratuberculosis (40). More widely used is ELISA, but usually in conjunction with other diagnostic methods such as fecal culture. Because ELISA is more sensitive than AGID, ELISA can detect subclinical infection more frequently. Estimates for the sensitivity and specificity of ELISA vary widely, depending upon the quality of reagents used in the assay. One major problem with ELISA is the variety of antigens used in the development of these tests, making comparisons between laboratories difficult. Reported sensitivity values for a commercial ELISA kit to detect paratuberculosis antibodies in the sera of cattle range from 15 to 57% for subclinically infected cattle shedding low numbers of organisms in their feces; the average is 88% for clinically infected cattle shedding high numbers of organisms (11, 47). Diagnostic specificity of this commercial kit is estimated to be 99% or greater. Preadsorption of test sera with *Mycobacterium phlei*, an environmental contaminant, prior to testing has markedly enhanced the specificity of the ELISA technique and was a critical step in the development of the commercial kit (62). Development of ELISA tests incorporating more specific antigens to *M. paratuberculosis* has resulted in an increase in the sensitivity of detection (70 to 80%) and a concomitant decrease in specificity (89 to 95%) (2, 52). The CF test is most frequently used to test cattle for import and export purposes. However, as with other serologic tests, the CF test lacks the necessary sensitivity to be used for definitive diagnosis of paratuberculosis infection and has lower specificity than AGID or ELISA.

Recognition that strong cell-mediated immunity in the subclinical stage of infection predominates led to the development of an assay to measure the release of interferon (**IFN**)-γ by cells that had been stimulated in culture with mycobacterial antigens (60). The amount of IFN-γ released is subsequently quantified in an ELISA using an enzyme-linked monoclonal antibody to bovine IFN-γ. The advantage of this test is its ability to detect subclinically infected animals in a herd, enabling producers to implement more stringent management regimens by segregating infected cattle from the remainder of the herd or by culling them (44).

Methods of detection for paratuberculosis infection have recently been developed using nucleic acid probes combined with polymerase chain reaction (**PCR**). The first DNA probe that was developed for detection of *M. paratuberculosis* DNA in fecal specimens lacked specificity because the probe also hybridized with DNA from *Mycobacterium avium* (22). More recently, other DNA probes have been developed that are specific for *M. paratuberculosis*. One probe is based upon partial sequence of an insertion element of *M. paratuberculosis*, IS900 (54). A DNA probe test kit based upon the IS900 sequence for diagnosis of *M. paratuberculosis* infection has been developed and licensed for sale in the US (IDEXX, Westbrook, ME). Studies (56) conducted to compare the DNA probe test kit with three different procedures for fecal culture indicate that about 60% of infected cattle detected by fecal culture can be detected using the DNA probe. Therefore, although highly specific for *M. paratuberculosis*, the DNA probe is unable to detect infected cattle that are shedding low numbers of organisms. Another probe that is specific for *M. paratuberculosis* is recombinant clone F57 (33), which is currently being used in some laboratories. A recent modification of the polymerase chain reaction to include two consecutive amplification reactions using nested primers markedly increased the sensitivity of this test (13). This method was able to detect 50 organisms/g of feces compared with 10^4 organisms/g for the commercial kit.

Treatment of Paratuberculosis

Treatment of paratuberculosis with antimicrobials is an expensive venture at best and is ineffective at worst. Standard antituberculosis drugs such as clofazimine, isoniazid, rifabutin, rifampin, and streptomycin have been tested both in vitro and in vivo for effects on *M. paratuberculosis* (46). Although many in vivo studies have demonstrated an improvement of clinical signs of paratuberculosis in treated animals, the organism could still be detected in the feces. Combination therapy with two or more of the aforementioned drugs has proven to be slightly more effective (15, 34, 41). A 60-d treatment of goats with paratuberculosis that used a combination of streptomycin, rifampicin, and levamisole markedly improved body weight gain, total serum protein, and globulin (15). Fecal shedding of the organism was not observed after treatment, and the organism was not detected in tissues post-mortem. Currently, antimicrobial therapy is not considered a viable option for treatment of paratuberculosis infection because of expense, because of the extended periods that drugs need to be administered, and because of disease recurrence after therapy is discontinued.

Vaccination

Since its introduction in 1926, vaccination has been a controversial method for control of paratuberculosis. Researchers have shown that vaccination with heat-killed or modified live preparations of *M. paratuberculosis* strain 18 has effectively reduced the incidence of clinical disease in dairy herds, sheep, and goats (14, 26, 55). In addition, the number of paratuberculosis organisms shed in the feces of vaccinated animals is reduced, thereby lessening the potential spread to other infected animals in the herd (26, 27). Economic losses from decreased production by infected animals have also been reduced by vaccination (53). However, the benefits of vaccination cannot be clearly distinguished from concomitant improvements in other management practices, such as animal placement, manure disposal, and general hygiene practices. The major disadvantages of a paratuberculosis vaccine include a positive antibody test, which may interfere with serological testing for paratuberculosis or tuberculosis; granulomatous lesions at the site of vaccination; and potential granulomatous reactions from self-injection (8, 42). Use of the vaccine is restricted in the US, thereby limiting the amount of data available on its capacity to prevent infection or the spread of disease. The vaccine has been more widely used in Europe and Latin America with demonstrable benefit.

The advent of new technology should make significant improvements possible to the products that are now available. Several laboratories are currently working with gene products that are specific for *M. paratuberculosis* and that may be feasible for a vaccine. To date, vaccine preparations for paratuberculosis generally comprise whole-cell suspensions or sonicated preparations of the bacterium. There is some

dispute over whether soluble protein preparations are effective for use in a vaccine. A new area of research to be considered is the use of naked DNA from *M. paratuberculosis* as a vaccine candidate. Preliminary studies with other bacterial organisms indicate that DNA vaccination may be an effective way to achieve protection.

Improved Management for Control of Paratuberculosis

One of the foremost considerations for a management program for control of paratuberculosis is proper manure disposal. A common flaw of many dairy operations is the use of the same skid loader for feeding and manure disposal (19). Cross-contamination of the feed is a major contributor to the spread of paratuberculosis, and feces are the major source of the causative organism. Use of improperly treated manure solids to fertilize pastures on the farm is another source of paratuberculosis infection because the organism can survive in the soil for up to a year. Young calves are the most susceptible group of animals in the herd, and calves should be removed from the dam immediately after birth (58). Because *M. paratuberculosis* is shed in the colostrum and milk of clinically infected cows, the caretaker should ensure that calves are fed uncontaminated colostrum and milk replacer to prevent infection. Segregation of infected animals from uninfected animals is a good idea at any age, and the manure of each group should be disposed of separately. Replacement heifers should be kept separate from other members of the herd until their infection status can be ascertained (36). Distinct watering sites must be available for infected and noninfected animals. Stagnant water sources are excellent reservoirs for numerous bacteria, and *M. paratuberculosis* has been found to survive in such water for long periods. The main advice for producers experiencing problems with Johne's disease is to "clean, clean, clean".

M. paratuberculosis: A Human Pathogen?

Recent evidence suggests that the etiological agent in Crohn's disease in humans, a severe inflammatory enteritis involving the terminal ileum, may be of mycobacterial origin (6, 49). Clinical studies have demonstrated the presence of several species of mycobacteria, including *Mycobacterium fortuitum*, *M. avium* ssp. *intracellulare*, *Mycobacterium cheloni*, and *Mycobacterium kansasii* in intestinal biopsy tissues from patients with Crohn's disease (6). More recently, *M. paratuberculosis* has been successfully isolated from patients with Crohn's disease (6, 38). Using the IS900 DNA probe, which is specific for *M. paratuberculosis*, workers (16, 17, 32, 38) have been able to identify the presence of paratuberculosis DNA in intestinal tissue from patients with Crohn's disease. Because the clinical symptoms of Crohn's disease closely mimic those found in animals with Johne's disease, a number of laboratories have proposed that *M. paratuberculosis* may be the causative agent of Crohn's disease (32, 38). However, an equivalent number of studies have been unable to demonstrate the presence of *M. paratuberculosis* DNA in tissue of patients with Crohn's disease (18, 35, 37, 61). Epidemiological evidence correlating exposure to *M. paratuberculosis* with incidence of Crohn's disease is not readily available. However, cows with clinical paratuberculosis do shed viable organisms in their milk at low concentrations (50 cfu/50 ml of milk) (48). In addition, in the United Kingdom, Millar et al. (31) recently demonstrated the presence of *M. paratuberculosis* DNA in milk samples obtained from retail markets. They also described identification of viable *M. paratuberculosis* from the pasteurized retail milk samples after long-term incubation ($\leq$ 40 mo). Interestingly, 9 of 18 milk samples that were previously positive for PCR and 6 of 36 milk samples that were previously negative for PCR were culture positive. Results from that study (31) have been highly controversial and suggest that current pasteurization techniques may not be adequate to kill *M. paratuberculosis* in raw milk. A number of laboratories have recently investigated optimal time and temperature combinations for heat inactivation of *M. paratuberculosis* in milk. Laboratory studies (7, 20) simulating either the standard holding method (63.5°C for 30 min) or the HTST method (71.7°C for 15 s) of pasteurization have demonstrated that a residual population of viable *M. paratuberculosis* survives after treatment of milk (7, 20). However, studies (45) conducted at the National Animal Disease Center using a laboratory-scale pasteurizer have demonstrated that raw milk inoculated with live *M. paratuberculosis* (10^4 or 10^6 cfu/ml) at 72°C for 15 s effectively killed all the bacteria (45). Those data are further corroborated by work (21) conducted in Australia using a small-scale commercial pasteurizing unit. The major difference between the test tube model and the laboratory-scale pasteurizer model was the static versus active flow of milk during heat treatment. Although this difference may not be the only reason for the discrepant results between the two experimental models, results suggest that turbulent flow of milk during the pasteurization process, such

as is achieved in commercial dairies, appears to be necessary to achieve complete inactivation of contaminating bacteria because organisms may clump more readily in a static environment and protect themselves from heat penetration. Although preliminary, those studies indicate that transmission of viable *M. paratuberculosis* from animals to humans via pasteurized dairy products in unlikely and minimizes its potential threat as a zoonotic agent in Crohn's disease.

CONCLUSIONS

There is a need for improved diagnostic tests for the detection of Johne's infection in cattle. Efforts need to be concentrated on the development of simple, rapid, noninvasive tests that can be performed by veterinarians or producers without expensive laboratory equipment. Further research to identify and characterize antigenic proteins that are specific for *M. paratuberculosis* is necessary for improved vaccines. In the interim, improvements in hygienic practices on the farm need to be implemented, and careful consideration needs to be given to manure disposal from infected animals. Further studies also need to be conducted to identify possible sources of *M. paratuberculosis* contamination for humans in the event that this organism is classified as a zoonotic agent.

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Viral Association with Crohn's Disease

M. S. H. Smith and A. J. Wakefield

Crohn's disease is an inflammatory disorder of the gastrointestinal tract, the cause of which remains unknown. Since the first description by Dalziel in 1913 (1), similarities between Crohn's disease and intestinal mycobacterial infection, particularly Johne's disease in ruminants, have been widely recognized (2, 3). After Mitchell and Rees demonstrated the transmission of granulomata from Crohn's disease by injecting intestinal homogenates into the footpads of mice (4), there followed many studies attempting to identify infective agents within the bowel of patients with Crohn's disease. Although *Mycobacterium paratuberculosis* has been identified in intestinal tissue from a proportion of patients with Crohn's disease, a convincing role for this agent in the aetiology of Crohn's disease has not been established (5). Likewise, extensive studies into bacterial (6–9) and viral (10) agents potentially associated with Crohn's disease have been inconclusive, although recent ultrastructural observations of viral particles within submucosal granulomata have added a new impetus to the search (11, 12).

This review examines the evidence for an association between Crohn's disease and viral infection from epidemiological studies, transmission and cell culture, specific immunological responses, ultrastructure and from molecular biological techniques.

Key words: Crohn's disease; virus; measles

(Annals of Medicine 25: 557–561, 1993)

Epidemiology

In a detailed case record analysis. Ekbom et al. studied 4000 patients with inflammatory bowel disease in the Uppsala health care region in Sweden (13). The authors found a higher incidence of inflammatory bowel disease in urban compared to rural counties and a higher age-specific incidence rate for those born between 1945 and 1954 compared to other years. This apparent birth cohort effect was accounted for by an excess of births in the first half of the year. The same group had previously shown perinatal viral illness to be a strong risk factor for the subsequent development of inflammatory bowel disease (14) and they suggested that the birth cohort effect may have resulted from viral infections acquired in the perinatal period. Wurzelmann et al. undertook a retrospective survey by questionnaire of patients with inflammatory bowel disease and found a general increase in the frequency of childhood infections in Crohn's disease compared with controls (15). They did not identify any difference in viral infections. In a similar study, Gilat et al. (56) found no difference in overall childhood infections but reported significantly more respiratory infections in patients prior to the time of diagnosis of inflammatory bowel disease. These retrospective surveys are likely to be considerably less sensitive than the Uppsala studies, since they are reliant on recall of distant events rather than data gathered prospectively. Further useful information may be gained from prospective cohort studies currently in progress. The evidence for a genetic element in Crohn's disease is conflicting (16, 17). A familial incidence is recognized (18) but the mode of inheritance is not known. Of particular interest is a recent study of two French families with a high incidence of Crohn's disease. Van Kruningen et al. reported Crohn's disease in non-sanguinous relations across two generations, suggesting an environmental rather than a genetic influence (19).

Transmission and Cell Culture Studies

In 1970, Mitchell and Rees injected intestinal and lymph node homogenates from a patient with Crohn's disease

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into the footpads of mice, and were able to reproduce non-caseating granulomata at the sites of injection and, in some cases, in the intestine (4). These changes were not seen in those animals injected with normal tissue. Similar results were obtained by Taub and Siltzbach (20) although Bolton et al., using bowel homogenates alone, were unable to produce granulomata in any of their subjects (21). A condition similar to Crohn's disease was also produced by injected filtrates of ileum from patients with Crohn's disease into rabbits (22). Subsequent studies also produced conflicting results (23–25). In order to clarify the issue, an independent survey of tissue obtained from four laboratories was undertaken (26). It was concluded that many of the granulomata identified were related to foreign bodies. At present, the capacity for Crohn's disease to be transmitted by tissue transfer remains unproven (27). These studies prompted an extensive search for infective agents associated with inflammatory bowel disease.

Early attempts to identify viral agents involved the inoculation of homogenates and filtrates of intestinal tissue from patients with Crohn's disease onto cultured cell lines (28, 29). A cytopathic effect was frequently observed and analysis of the physicochemical characteristics of the agents involved suggested that the effect was caused by small RNA viruses such as picornaviruses or reovirus-like agents (29, 30). An independent study of specimens from one of the laboratories concerned identified *Mycoplasma* contamination in two out of five cultures (31). Later work showed that a cytopathic effect could be produced by the inoculation of normal bowel onto cultured cells and that this could not be passaged (32, 33). The effects observed were thought to be due to tissue cytotoxins (34, 35). Although *Mycoplasma* contamination and tissue cytotoxins may have caused cytopathic effects in some of the previous studies, they would not have explained many of the physicochemical ultrastructural findings.

Ultrastructure

Gitnick et al. reported the presence of viral agents in ileal filtrates from patients with Crohn's disease that had been grown in continuous rabbit ileum cell culture (29). Viral particles that were consistent in appearance with picornaviruses were identified by electron microscopy. No viruses were cultured from control tissue. Whorwell et al. isolated agents from intestinal tissue of patients with Crohn's disease which were more in keeping with reoviruses (30). Farmer et al., using similar techniques, found cytomegaloviruses (CMV) in one out of four specimens from patients with Crohn's disease and three out of six specimens from patients with ulcerative colitis (36). Yoshimura et al. were able to produce cytopathic effects in cell culture but could not identify any features compatible with viral infection by electron microscopy (32). The major limitation of ultrastructural examination in the search for viruses in inflammatory bowel disease is that such viruses need to be present in very large numbers in order to be detected. Since the features of Crohn's disease appear to be cell-mediated, it is possible that the putative infective agent need not be present in high titre and so may be missed by these techniques. Working on the hypothesis that Crohn's disease develops from an intestinal granulomatous vasculitis (37), Wakefield et al. focused their search on the submucosal microvascular endothelium and granulomata. They identified endothelial cell cytoplasmic inclusions highly suggestive of paramyxovirus nucleocapsids (11). Similar findings were later observed by Knibbs et al. (12). Further characterization of these particles by immunoelectron microscopy is currently in progress.

Immunology

Serological surveys of patients with inflammatory bowel disease have been performed by a number of groups. In 1973, Farmer et al. demonstrated a higher frequency of CMV antibodies in patients with ulcerative colitis compared with Crohn's disease and controls (36). Swarbrick et al. confirmed this finding but found that anti-CMV titres did not rise during acute attacks (28). In contrast, Kangro et al. found that disease relapse was preceded by serological evidence of viral or mycoplasma infection in 24% of children with inflammatory bowel disease (39). Apart from the findings in relation to CMV, the prevalence of antibodies against other herpesviruses, picornaviruses, reoviruses, paramyxoviruses, orthomyxoviruses and adenoviruses in Crohn's disease appear to be no different from controls (36, 40, 41).

Virus-specific cell-mediated immunity has not been reported in Crohn's disease. Chiba et al. assessed cell-mediated immunity to agents in Crohn's disease intestinal tissue causing *in vitro* cytopathic effects (42). No specific immune reactivity was seen by any of the assays used (indirect immunofluorescence, lymphocyte transformation and cytotoxicity). However, since only peripheral mononuclear cells were tested, responses mediated by intestinal mononuclear cells would not have been detected.

Molecular Biology

Virus isolation from the bowel by cell culture is a relatively insensitive method of detection.

The techniques of nucleic acid hybridization and polymerase chain reaction (PCR) analysis allow the detection of nucleic acid targets which may be present in very low numbers (43). In 1977, Roche and Huang analysed intestinal tissue from patients with inflammatory bowel disease for the presence of CMV using Southern blot and *in situ* hybridization (44). CMV was not detected in three cases of Crohn's disease and was found in only one out of nine cases of ulcerative colitis. The same group were unable to detect adenovirus DNA in intestinal tissue from patients with Crohn's disease and ulcerative colitis by Southern blot hybridization (45). Recently, Wakefield et al. reported their analysis of intestinal tissue

from patients with Crohn's disease and ulcerative colitis for the presence of herpesvirus DNA by nested PCR (46). They found a greater prevalence of both CMV DNA and Epstein-Barr virus (EBV) DNA in inflammatory bowel disease compared to controls. In addition, DNA from two or three viruses out of CMV, EBV and human herpesvirus 6 was more commonly present together in intestinal tissue from patients with ulcerative colitis compared to Crohn's disease or controls. If these viruses are aetiologically associated with ulcerative colitis, this may indicate that viral superinfection occurs in ulcerative colitis, leading to herpesviral reactivation and disease relapse, in the manner suggested by Kangro et al. (39). In relation to the findings of paramyxovirus-like particles in foci of granulomatous and lymphocytic vasculitis in Crohn's disease, Wakefield et al. probed tissue sections with a biotinylated riboprobe specific for negative-stranded (genomic) measles virus RNA (11). By this method, measles virus RNA was identified in all 10 cases of Crohn's disease examined and, specifically, within vascular endothelial cells associated with foci of inflammation in nine of 10 cases of Crohn's disease. Vascular staining was seen in four of 10 cases of ulcerative colitis although this bore no relation to inflammation. Similarly, vascular staining was seen in three of 10 non-inflammatory controls. Positive staining of the sections from patients with Crohn's disease was also obtained by immunohistochemistry using a monoclonal antibody specific for measles nucleoprotein. These results support an association between measles virus and Crohn's disease and suggest that persistent enteric measles infection may often be present in the general population.

In assessing the prevalence of viral infections and their importance to the aetiology and pathogenesis of Crohn's disease, these approaches raise a number of problems.

1. The significance of the agents identified may be hard to determine since many bacteria and viruses are present in normal intestine (46, 47).
2. Bacterial overgrowth is a well-recognized feature in Crohn's disease (48, 49) and the same may be true for enteric viruses, although there is no evidence for this at present.
3. Mucosal damage as a result of intestinal inflammation may allow invasion by micro-organisms as a secondary event.
4. If Crohn's disease results from a defective intestinal immune response triggered by an enteric infection early in life, the original pathogen need not be present once the disease process is established.
5. In contrast, if Crohn's disease results from an ineffective immune response to a persistent microbial infection, the infective agent may exist as a defective form capable of evading immune surveillance but unable to replicate in cell culture (50).
6. Viruses known to cause persistent infection commonly persist in lymphocytes (51) and may persist in normal individuals (52). Isolation of such agents in intestinal tissue affected by Crohn's disease may simply reflect infiltration by infected inflammatory cells.
7. Relapse in inflammatory bowel disease may be precipitated by infection although such infective agents are unlikely to be involved in the initial disease process (39).

At present, little is known about the prevalence or significance of persistent viral infection in the normal or diseased individual, in much the same way as we are still at an early stage in the study of 'normal' intestinal inflammation. In one of the best documented human examples of an organ-specific disease caused by a persistent virus, subacute sclerosing panencephalitis, the relative importance of host susceptibility and virus strain to the pathogenesis of the disease is still unknown (53), although exposure to measles virus early in life is a well-recognized risk factor (54). To date, there has been only one study which has attempted to detect viral persistence in the intestine in Crohn's disease (55). Phillpotts et al. examined cultured cells derived from trypsinized intestinal tissue from Crohn's disease by electron microscopy and by indirect immunofluorescence for CMV. Interferon production, changes in lectin binding and reverse transcriptase activity were also measured. No evidence for persistent viral infection was found although the technique inevitably selected the most robust cells, such that virus-infected cells may not have survived.

Clearly, these difficulties will hamper further progress in elucidating the relationship between viral agents and Crohn's disease. What is the most fruitful approach? The key probably lies in the submucosal granuloma and its associated vascular tissue. Macrophages form granulomata when presented with antigens which they cannot eliminate: if Crohn's disease is caused by a virus, the granuloma is likely to represent the site of viral antigen presentation. Since 85% of granulomata in Crohn's disease appear to have a vascular origin (37), the principal site of antigen presentation may be the vascular endothelium. An extensive immunological, ultrastructural and molecular examination of Crohn's granulomata and associated vascular structures is therefore warranted. If a single agent causes Crohn's disease, this approach offers the best chance of finding it.

We wish to thank all members of the Inflammatory Bowel Disease Study Group for their continuing efforts and the Wellcome Trust, National Association for Colitis and Crohn's Disease and Crohn's and Colitis in Childhood for their support.

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Ultrastructural immunogold localization of subcellular sites of TNF-α in colonic Crohn's disease

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Abstract: Tumor necrosis factor-α, a proinflammatory cytokine, might have an important role(s) in initiating, modifying, and/or sustaining chronic inflammatory processes such as those that characterize Crohn's disease, an inflammatory bowel disease of unknown etiology. We used an immunogold ultrastructural morphometric approach to localize tumor necrosis factor-α in colonic Crohn's disease biopsies. Tumor necrosis factor-α was present in seven cell types (fibroblasts, eosinophils, mast cells, macrophages, colonic epithelial absorptive cells, Paneth cells, neutrophils). Tumor necrosis factor-α–containing subcellular organelles included lipid bodies (fibroblasts, eosinophils, macrophages, mast cells, colonic epithelial cells, neutrophils), secretory granules (eosinophils, Paneth cells), phagolysosomes (macrophages, colonic epithelial cells), and Golgi structures and vesicle membranes (neutrophils). A gradient of extracellular tumor necrosis factor-α immunoreactivity surrounded eosinophils, mast cells, and macrophages. *P* values of gold counts/μm² were significant for all cells, organelles, and extracellular spaces measured, and all positive structures significantly exceeded the background labeling density/μm². Specificity controls (normal rabbit serum, tumor necrosis factor-α–absorbed primary antibody) either failed to label these sites or gave markedly reduced specific tumor necrosis factor-α labeling, respectively. These findings represent the first ultrastructural localization of the subcellular sites of TNF-α in vivo in seven cell lineages in human colonic tissues. *J. Leukoc. Biol.* **58: 284–298; 1995.**

Key Words: eosinophil · mast cell · macrophage · lipid bodies · secretory granules · phagolysosomes

INTRODUCTION

Tumor necrosis factor-α (TNF-α), originally named for its ability to induce hemorrhagic necrosis of malignant tumors in mice, is now a well-known mediator of inflammation, cytotoxicity, and cachexia (reviewed in refs. 1–4). TNF-α, a proinflammatory cytokine, participates in inflammatory reactions through direct and indirect effects that contribute to the recruitment and activation of inflammatory cells. TNF-α interacts with other cytokines [1, 2] in inflammatory reactions and induces a catabolic state by interfering with lipid metabolism [2]. TNF-α suppresses lipogenic enzymes, such as lipoprotein lipase and fatty acid synthase, delays differentiation of mature adipocytes, and stimulates the loss of stored lipids in adipocytes (reviewed in ref. 2). TNF-α has also been linked to the metabolism of arachidonic acid, a pathway that produces lipid mediators of inflammation, such as prostaglandins and leukotrienes [3]. These studies include evidence that TNF-α synthesis is down-regulated by prostaglandins [4, 5], a process that may be diminished or lost in certain types of injury [6]. Macrophage TNF-α production can be modulated by exposure to fatty acids [7], and TNF-α can potentiate phospholipase A_2–stimulated arachidonic acid release and formation of prostaglandin E_2 from cultured intestinal epithelial cells in vitro [8]. TNF-α is also known to stimulate prostaglandin synthesis by macrophages [9, 10].

There is increasing evidence that both TNF-α [11–19] and eicosanoids [20–22] play a role in inflammatory bowel disease. Immunohistochemistry, in situ hybridization, and spot ELISAs of isolated intestinal cells revealed more TNF-α protein, mRNA, and secretion in the intestines of patients with Crohn's disease (CD) or ulcerative colitis than in controls [11–15]. TNF levels were also reported to be increased in the serum of CD patients [12]. Peripheral blood monocytes of CD patients were found to secrete increased amounts of TNF-α after lipopolysaccharide challenge but not spontaneously [16]. TNF-α levels measured in stool samples of children with CD correlated with disease activity [17, 18], and TNF-α monoclonal antibody therapy caused a 3-month remission in a girl with cachexia, fever, and therapy-resistant CD of the colon [19]. On the other hand, several reports using polymerase chain reaction amplification of total mRNA in biopsy samples did not demonstrate a difference in TNF-α RNA levels compared to controls [23, 24]. However, in one of these reports, only two colonic biopsies (of inactive CD) were examined, the remainder being of small intestinal sites [23].

A variety of techniques have been used to document TNF-α synthetic and secretory capacity in individual cells. These techniques include light microscopic immunohistochemical methods to detect protein, light microscopic in situ hybridization to detect messenger RNA, biochemical assays of secretory products from purified cells, and immunogold detection of protein by electron microscopy. In aggregate, these studies implicate macrophages [25, 26], fibroblasts [27, 28], Paneth cells [26, 29], epithelial cells [30–35], neutrophils [36–38], eosinophils [26, 39, 40], and mast cells [41–44] (and reviewed in refs. 45, 46) as potential sources of TNF-α. We have found that ultras-

Abbreviations: CD, Crohn's disease; HBSS, Hanks' balanced salt solution; HES, hypereosinophilic syndrome; NSS, normal swine serum; PMD, piecemeal degranulation; TNF-α, tumor necrosis factor-α.

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Received March 3, 1995; accepted May 15, 1995.

tructural immunogold analysis of specially fixed and processed materials and appropriate controls serves to identify precisely those cells and subcellular organelles that contain TNF-α [40, 44] (W.J. Beil et al., unpublished data). Because all of these previous studies were done on isolated, partially purified cells and because of the recent implication of TNF-α in the pathophysiology of CD, we examined in colonic biopsies the substructural sites and cell types that contain TNF-α in vivo in this complex inflammatory disease of unknown etiology (reviewed in ref. 47).

MATERIALS AND METHODS

Patients and samples

Multiple colon biopsies were obtained during colonoscopy from six patients with CD. In **Table 1**, we list the relevant clinical and biopsy information for these patients. This includes the patients' age and sex, the duration of CD, the medications being taken at the time of sample collection, previous surgical procedures involving gastrointestinal organs, associated clinical diagnoses, and the presence or absence of CD, as assessed both by gross inspection at endoscopy and by light microscopic analysis. The diagnosis of CD was established according to accepted clinical and pathological criteria [47–49]. A total of nine biopsies from six patients (for whom biopsies were done for diagnosis and/or planning of management) were examined for TNF-α localization by electron microscopy. Informed consent was obtained from all patients, in accord with a protocol approved by the Beth Israel Hospital Institutional Human Research Review Committee.

Fixation and processing for electron microscopic studies

Endoscopy samples were fixed by immersion, less than 1 min after excision, in mixtures of freshly prepared aldehydes in 0.1 M sodium cacodylate buffer, pH 7.4, containing 0.025% $CaCl_2$, for 2 h at room temperature. Following immersion fixation, samples were washed in 0.1 M sodium cacodylate buffer, pH 7.4, 4°C. All samples were postfixed by immersion for 1 h in 2% OsO_4 in 0.2 M Sym-collidine buffer at 20°C. Subsequently, the samples were washed in three changes of sodium maleate buffer, pH 5.2, stained en bloc in 2% uranyl acetate in sodium maleate buffer, pH 6.0 [50], washed 3× in sodium maleate buffer, pH 5.2, dehydrated in a graded series of alcohols, and infiltrated and embedded in a propylene oxide–Epon sequence. Eighty- to 100-nm thin sections were prepared and placed on 200-mesh gold grids (Ted Pella, Tustin, CA).

Immunogold staining for electron microscopy

Gold grids containing freshly cut thin sections were treated with the following sequential steps at room temperature [40, 44]: (1) 4% sodium metaperiodate in distilled water, 15 min; (2) wash once in Hanks' Ca_2Mg-free balanced salt solution (Biosciences, Lenexa, KS) (HBSS) containing 0.1% Triton X-100 (Sigma, St. Louis, MO) for 15 min; (3) 5% normal swine serum (NSS) in HBSS containing 0.1% Triton X-100, 30 min; (4) incubation for 14 h in a 1:30 dilution of the primary, polyclonal, rabbit anti-human TNF-α antibody (Genzyme, Boston, MA; lot numbers B3326 and B2439) in 2.5% NSS in HBSS with 0.1% Triton X-100; (5) wash in HBSS plus 0.1% Triton X-100, 15 min; (6) incubation for 40 min in 1:60 dilution of the secondary antibody, swine anti-rabbit immunoglobulin conjugated to 10-nm gold (Dako, Carpenteria, CA) in HBSS plus 0.1% Triton X-100 plus 2.5% NSS; (7) wash for 30 min in HBSS plus 0.1% Triton X-100; (8) wash for 10 min in distilled water.

Three controls for specificity were done as follows: (1) omission of the primary (anti-TNF-α) antibody from the above staining sequence; (2) substitution of nonimmune rabbit serum [dilutions of 1:10, 1:20, or 1:30 (Jackson, West Grove, PA; Vector, Burlingame, CA)] for the primary antibody; and (3) substitution of TNF-α–absorbed or sham-absorbed antibody for the primary TNF-α–specific antibody. The TNF-α–absorbed antibody was prepared as follows: 2 μl of anti-human TNF-α antibody was placed in 50 μl of phosphate-buffered saline, pH 7.4, containing 2.5% NSS, 0.1% Triton X-100 and 1% (w/v) (10 μg/ml) rhTNF-α (Genzyme, Boston, MA; lot B31024) and incubated on a shaker for 2 h at 20°C. Another 2 μl of anti-human TNF antibody was placed into the same solution (without rhTNF) to prepare a sham-absorption control reagent [40, 44].

Following the above procedures, all sections on gold grids were contrasted with a methyl cellulose–uranyl acetate stain as described [51] and examined in a Philips 300 transmission electron microscope. Additional thin sections (of material not stained with the immunogold procedures) were stained with lead citrate and examined in the electron microscope.

Statistical analysis

Labeling density was determined by counting gold particles of defined structures (see **Table 2**) on randomly obtained electron micrographs of 389 cells printed at a final magnification of ×27,000. Individual organelles were measured by tracing their outlines and digitizing them with a computer equipped with IM-Series, version 3.46p software (Analytical Imaging Concepts). Labeling density was then expressed as number of gold grains per μm^2. To measure TNF-α in the vicinity of particular cells (e.g., as a result of TNF-α secretion), an area not exceeding 2 μm^2 of surrounding extracellular space directly adjacent to defined cell types (see Table 2) was used for measuring gold densities, using an analysis like that described above for cell organelles. Spaces between two or several cells were avoided. Gold densities of relevant intracellular structures and extracellular spaces (Table 2) were subjected to statistical analysis using the ANALYSER system. After checking for normal distribution, P values of data groups of sizes $n = 9–18$ were analyzed with the Newman-Keuls test for normally distributed data and the unblocked Kruskal-Wallis test for data that were not normally distributed. P values $<.05$ were considered to represent statistically significant values.

RESULTS

Sections from mucosal biopsies contained epithelium and lamina propria. Generally, submucosa and tunica muscularis were absent from these endoscopic biopsies.

TABLE 1. Crohn's Disease Colonic Biopsy Samples Used to Demonstrate TNF-α by Ultrastructural Immunogold Staining: Clinical Data

						Specimen(s)	
Case	Sex/age	Duration (years)	Medications at time of sample	Previous gastrointestinal surgery	Other clinical diagnoses	Endoscopy, uninvolved (UN), involved (I)	Pathology, uninvolved (UN), involved (I)
1	F/34	11	0	0	0	UN	UN
2	M/40	20	Ursodiol	Ileocolectomy	Colonic adenocarcinoma; primary sclerosing cholangitis	UN	I
3	M/45	19	Mesalamine, Prednisone	0	0	UN, I	UN, UN
4	M/73	7	6-Mercaptopurine	0	0	UN, I	UN, I
5	M/65	7	Ursodiol, sulfasalazine	0	Primary sclerosing cholangitis; pancreatitis; rectal fissures	UN, I	UN, I
6	M/27	18	Prednisone, mesalamine, azathioprine	Ileocecal resection	Perirectal abscesses	UN	I

TABLE 2. Immunogold Subcellular Localizations of TNF-α in Colonic Biopsies of Crohn's Disease Patients

Case	Cell	Organelle	ECS[a]	Number of cells counted			Number of organelles counted			Area (μm^2)			Gold label density (gold/$\mu m^2 \pm SEM$[b])			% reduction[f]	P values[d]	
				TNF[e]	NRS[g]	AC[h]	TNF	NRS	AC	TNF	NRS	AC	TNF	NRS	AC	BG[i]		TNF vs. NRS	TNF vs. AC
6	EOS[j]	LB[j]		12	10	9	12	10	9	4.5	3.4	4.8	36 ± 23	3 ± 2	2 ± 3	0.4	94	.0002	.0001
3,5,6		2° Granule		17	18	11	101	59	84	35	27	31	34 ± 9	1 ± 2	2 ± 1	0.1	94	<.0001	<.001
6			2 μm around EOS	11	9	10				57	45	55	18 ± 12	0	2 ± 2	0.1	87	.0059	.01
6	MC[k]	LB		18	12	11	58	23	12	22	9	5	29 ± 10	1 ± 2	6 ± 9	0.1	79	<.0001	.038
3,5,6			2 μm around MC	12	9	18				58	38	65	14 ± 7	1 ± 1	2 ± 2	0.4	84	.0003	.0031
3,5,6	MA[l]	LB		14	15	8	17	15	10	11	5.8	4	34 ± 15	0	2 ± 3	0.4	94	<.0001	.0002
		Phagolysosome		10	13	11	34	60	58	79	70	42	15 ± 6	1 ± 1	3 ± 3	0.4	79	<.001[m]	<.001[m]
			2 μm around MA	9	9	9				37	45	58	11 ± 3	1 ± 1	2 ± 1	0.4	81	<.01[m]	<.01[m]
5	CEC[n]	LB		8	18	14	48	70	48	38	17	11	18 ± 9	2 ± 3	1 ± 2	0.1	96	<.0124	<.0007
3,5,6	Fibroblast	LB		9	10	7	9	10	7	3	3	2.7	44 ± 27	2 ± 4	6 ± 5	0.4	86	.0002	<.001[m]
2	Paneth cell	Secretory granule		13	14	11	24	28	19	78	55	31	10 ± 6	0	1 ± 1	0.03	92	<.001	<.0101

[a]ECS = extracellular space.
[b]SEM = standard error of the mean.
[c]% reduction of TNF label density by absorption control.
[d]Significant P values for Kruskal-Wallis and Newman-Keuls tests are <.05.
[e]TNF = primary antibody to tumor necrosis factor-α.
[f]NRS = normal rabbit serum.
[g]AC = absorption control.
[h]BG = background.
[i]EOS = eosinophil.
[j]LB = lipid body.
[k]MC = mast cell.
[l]MA = macrophage.
[m]P values done by Newman-Keuls test; remainder done by Kruskal-Wallis test.
[n]CEC = colonic epithelial cell.

Eosinophils

Tissue eosinophils (**Figs. 1 and 2**)* in colonic biopsies of patients with Crohn's disease conformed to previous reports of the ultrastructural pathology of these cells in this disease (reviewed in refs. 47, 52). That is, they generally showed morphological signs of activation or of cell death. Tissue eosinophils that were activated showed changes in the number and contents of their secretory granules. Generally, specific bicompartmental granules were decreased and/or showed alterations in the normal contents of either or both compartments of these granules (reviewed in ref. 52). In contrast, lipid bodies (nonmembrane-bound, osmiophilic cytoplasmic organelles known to contain arachidonic acid [53–55], cyclooxygenase [54, 56, 57], and 5-lipoxygenase [58]) were increased (Fig. 2A, B, C, E). Actual extrusion of membrane-free secretory granules, recently visualized in eosinophils present in biopsies of human enteric tissues [59], was absent in activated eosinophils in the Crohn's disease colonic biopsies studied here. However, small cytoplasmic vesicles were increased (Figs. 1 and 2). Necrosis of tissue eosinophils, characterized by release of membrane-bound granules and destruction of nuclear and cytoplasmic structures, resembled that of previous reports (reviewed in ref. 52).

In all biopsies, the specific secondary granules of most eosinophils contained TNF-α (Figs. 1A, C and 2A, D, E). Labeling was present in both compartments of the secondary granules (Figs. 1 and 2). When evidence of piecemeal losses from secondary granule matrix and/or core compartments was present (Figs. 2D, F, G) [60, 61], TNF-α immunoreactivity was sometimes still present in the granules (Fig. 2D), although reduced, compared to fully intact granules. Also, the distribution of TNF-α label in these altered granules was heterogeneous and was located over the entire granule, unlike that in granules with well-preserved matrix and crystalline core domains, where most label was in the matrix. Intragranular irregular masses of remaining core material (Fig. 2D) and intragranular vesicles within electron-lucent matrix compartments (Fig. 2E) contained TNF-α, as did perigranular cytoplasmic vesicles (Fig. 2A) in these morphologically activated cells. Cytoplasmic lipid bodies in activated tissue eosinophils also contained TNF-α (Fig. 2).

Immunocytochemical controls, using nonimmune rabbit serum (Table 2) rather than specific antibody to TNF-α, showed no significant labeling of these eosinophil substructural organelles ($P = .0002$ for lipid bodies; $P < .0001$ for granules, vs. values for structures labeled with antibody to TNF-α) (Figs. 1D and 2B, G). Preadsorption of the specific antibody to TNF-α with rhTNF-α caused significant reduction of labeling of the TNF-α positivity of organelles in activated tissue eosinophils (Figs. 1B and 2C, F; Table 2). For example, label density for lipid bodies was reduced by 94% ($P = .0001$) and, for secondary gran-

*Figures 1–4 and Figure 7B are from case 6, a colon biopsy from an area that appeared uninvolved by Crohn's disease by endoscopy but that showed involvement by histologic examination; Figures 5 and 6 are from case 5, a biopsy of colon from an area that appeared involved by Crohn's disease; Figure 7A,C,D is also from case 5, but from a biopsy of uninvolved colon; Figures 8–10 are from case 2, a colon biopsy from an area that appeared uninvolved by endoscopy but that showed histologic evidence of involvement.

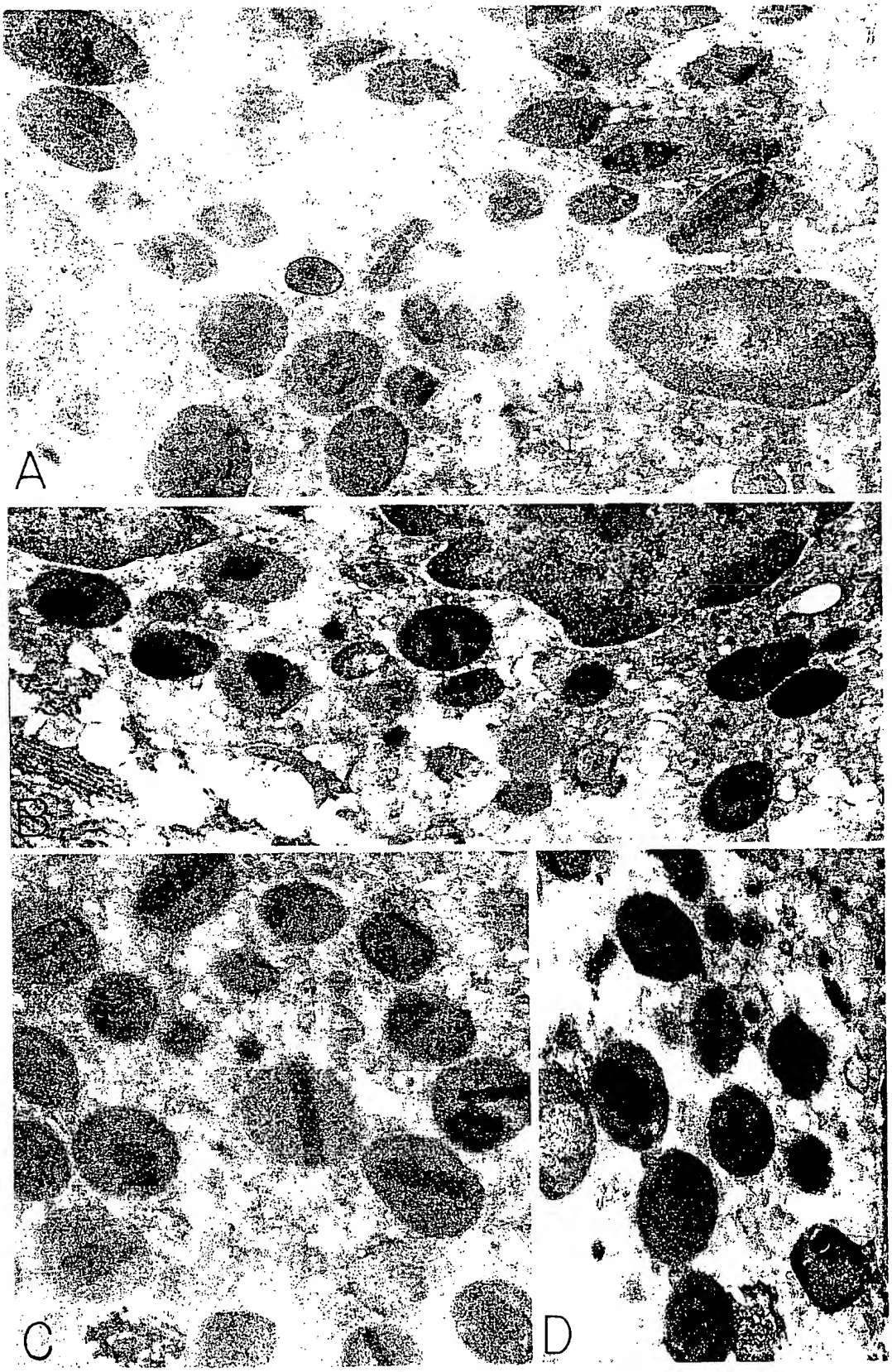

Fig. 1. Eosinophils show gold-labeled secondary granules after staining with the primary antibody to TNF-α (A). Substitution of TNF-α-absorbed primary antibody abrogates this label (B), whereas label persists after sham absorption of the antibody to TNF-α (C). Note that most of the gold particles associated with the unaltered, bicompartmental eosinophil secondary granules (A, C) are located over the outer, less dense matrix compartment. There is no eosinophil secondary granule gold labeling in specimens in which a nonimmune rabbit serum was substituted for the TNF-α-specific primary antibody (D). (A–D) ×20,500 (before 7% reduction).

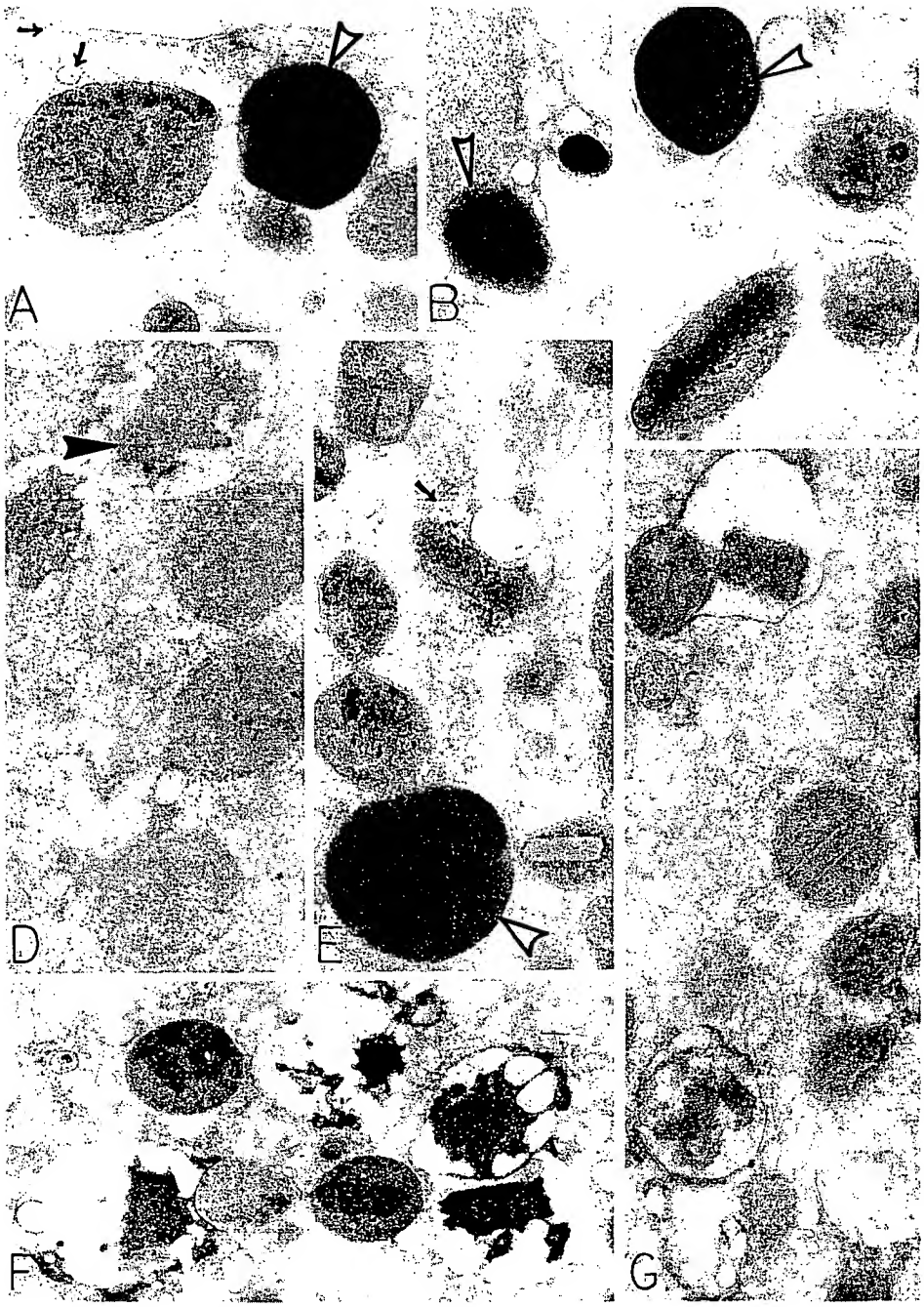

Fig. 2. Eosinophils show gold-labeled lipid bodies (open arrowheads) and secondary granules when stained with the antibody specific for TNF-α (A, D, E); some perigranular vesicles (A, arrows) also exhibit gold label. When nonimmune rabbit serum (B, G) or TNF-α–absorbed (C, F) primary antibody to TNF-α was used instead of specific antibody, lipid bodies (open arrowheads), secondary granules, and cytoplasmic vesicles did not stain. Eosinophil secondary granules that showed partial losses from matrix and/or core compartments (D, E) were also labeled with gold, indicating the presence of TNF-α. In some cases, the remaining, irregular core material contained TNF-α (D, filled arrowhead). Some altered granules had vesicles within their matrix compartments; these structures also contained TNF-α immunoreactivity (E, arrow). (A) ×36,000; (B) ×22,000; (C, D, G) ×31,000; (E) ×32,000; (F) ×30,000 (before 25% reduction).

ules, also by 94% ($P < .001$), vs. values for structures labeled with antibody to TNF-α.

We also measured gold densities/μm^2 in no greater than a 2-μm linear distance around tissue eosinophils that were located in areas away from other secretory cells. This analysis revealed a 180-fold increase in label (indicating TNF-α) compared to the background label of the same Epon sections (Table 2). This extracellular perieosinophil label for TNF-α showed an 87% reduction in the adsorption control ($P = .01$), and nonimmune rabbit serum did not significantly label this area ($P = .0059$ vs. label in sections stained with anti-TNF-α) (Table 2).

The relative density of TNF-α in subcellular sites of activated tissue eosinophils is shown in **Table 3.** Eosinophils were the second most highly labeled cells (of the six TNF-α–positive cells that were evaluated morphometrically). Within eosinophils, the density of TNF-α label of lipid bodies exceeded that of granules; and either of these values exceeded that within the 2-μm extracellular space surrounding secreting eosinophils, which showed a 180-fold increase over background Epon in the same sections. Of the lipid bodies evaluated in five cell types, TNF-α density of eosinophil lipid bodies exceeded all but that of fibroblast lipid bodies; of secretory granules (or macrophage phagolysosomes) in four cell types, eosinophil secondary granules contained the highest label for TNF-α. The relative density of TNF-α label in the extracellular space around eosinophils exceeded that surrounding mast cells or macrophages, using a similar morphometric analysis for relative densities of TNF-α specific gold label (Table 3).

TABLE 3. Rank Order of TNF-α Label Density in Cells and Their Organelles and Adjacent Extracellular Space in Colonic Biopsies of Crohn's Disease Patients

Rank order[a]	Cells (gold/μm²)	Eosinophils (gold/μm²)	Mast cells (gold/μm²)	Macrophages (gold/μm²)	ECS (gold/μm²)
1	Fibroblast (44)	LB[b] (36)	LB (29)	LB (34)	Eosinophil (18)
2	Eosinophil (35)	G (34)	ECS (14)	PL (15)	Mast cell (14)
3	Mast cell (29)	ECS (18)	G (0)	ECS (11)	Macrophage (11)
4	Macrophage (24.5)			L (0)	
5	Colonic epithelial cell (18)				
6	Paneth cell (10)				

	LB (gold/μm²)	G, PL, L (gold/μm²)	LB, G, PL, L (gold/μm²)
1	Fibroblast (44)	Eosinophil (34)	Fibroblast LB (44)
2	Eosinophil (36)	Macrophage PL (15)	Eosinophil LB (36)
3	Macrophage (34)	Paneth cell (10)	Eosinophil G (34); macrophage LB (34)
4	Mast cell (29)	Mast cell (0)	Colonic epithelial cell LB (18)
5	Colonic epithelial cell (18)	Macrophage L (0)	Macrophage PL (15)
6			Paneth cell G (10)
7			Mast cell G (0); macrophage L (0)

[a]Rank order, 1 > 7.
[b]Abbreviations: LB = lipid body; G = granule; ECS = extracellular space; PL = phagolysosome; L = lysosome.

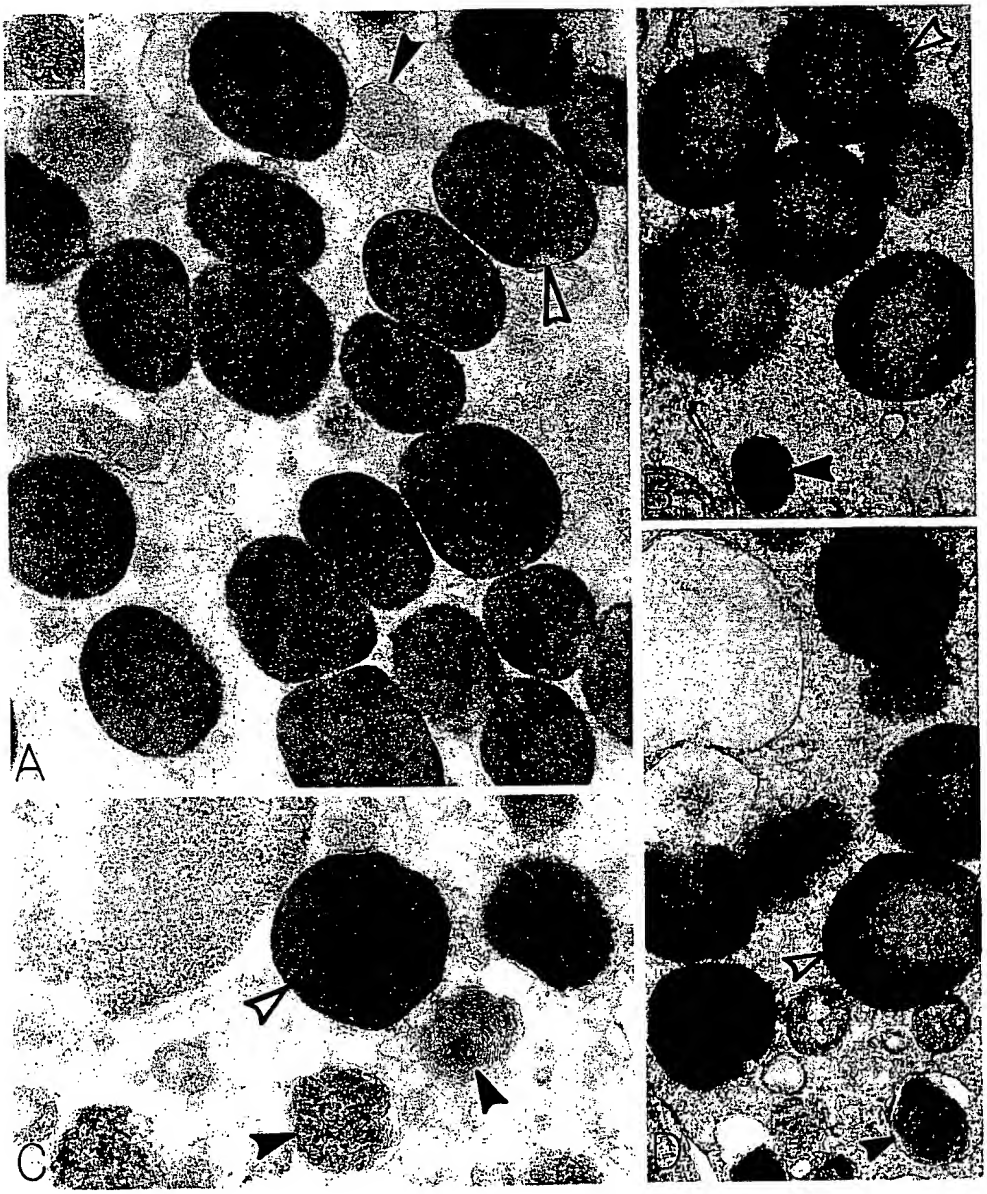

Fig. 3. Mast cells demonstrate TNF-α label in numerous large, nonmembrane-bound lipid bodies (A, open arrowhead). The smaller secretory granules do not label above background (A, filled arrowhead and inset). Lipid bodies (open arrowheads) and granules (filled arrowheads) were negative in nonimmune rabbit serum (B, D) and TNF-α absorption (C) controls. (A, C and inset) ×34,000; (B, D) ×32,000 (before 25% reduction).

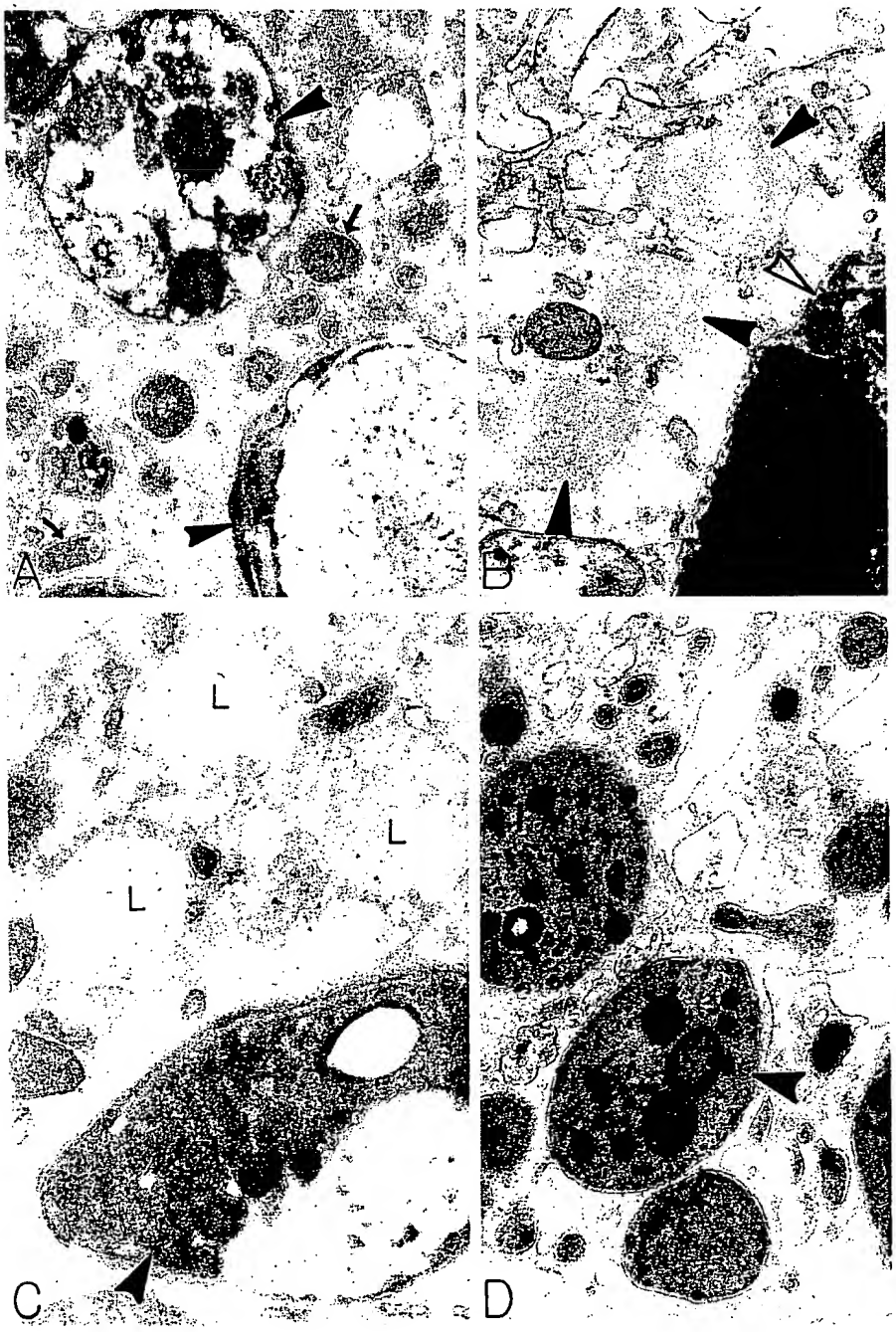

Fig. 4. Macrophages show TNF-α–positive phagolysosomes (filled arrowheads) (A, C) and lipid (L) bodies (C). Note that nonmembrane-bound macrophage lipid bodies are nearly electron lucent in contrast to those in eosinophils (Fig. 2) and mast cells (Fig. 3). Some cytoplasmic label is also seen (A). Primary lysosomes are not labeled (arrows) (A). Lipid bodies (filled arrowheads) and a phagolysosome (open arrowhead) in nonimmune rabbit serum (B) and phagolysosomes (filled arrowhead) in TNF-α absorption (D) controls are negative. (A) ×30,000; (B) ×28,000; (C) ×29,000; (D) ×35,000 (before 25% reduction).

Mast cells

Mast cells were also evaluated for the presence of TNF-α in Crohn's disease colonic biopsies. Generally, these mast cells exhibited evidence of activation, characterized by increased numbers of large, round, nonmembrane-bound, osmiophilic lipid bodies—structures that are known to be prevalent in human mast cells in several sites and in many diseases (reviewed in ref. 62) and that contain arachidonic acid [63, 64] and cyclooxygenase [65]. The secretory granules that we evaluated in these cells generally were of the scroll-containing ultrastructural pattern [66] (and reviewed in ref. 62) (an analysis of mast cells exhibiting the ultrastructural morphology of piecemeal degranulation, characterized by losses of dense granule materials and retention of the granule containers in the cytoplasm, is not included in the morphometric counts of this study [67]).

Lipid bodies in the mast cells that we studied contained TNF-α (**Fig. 3A**); secretory, scroll-filled granules did not label (Fig. 3A) above the Epon background. For example, there was a 290-fold increase in gold label in lipid bodies over the Epon background of the same sections (Table 2). The extracellular tissue space around mast cells also contained TNF-α, a 35-fold increase in gold density compared to Epon background (Table 2).

Immunogold specificity controls for TNF-α in mast cells were evaluated (Table 2 and Fig. 3B, C, D). Substitution of nonimmune rabbit serum for specific primary antibody to TNF-α failed to label either mast cell lipid bodies (Fig. 3B, D) ($P < .0001$) or the extracellular space around mast cells ($P = .0003$). Absorption of the specific

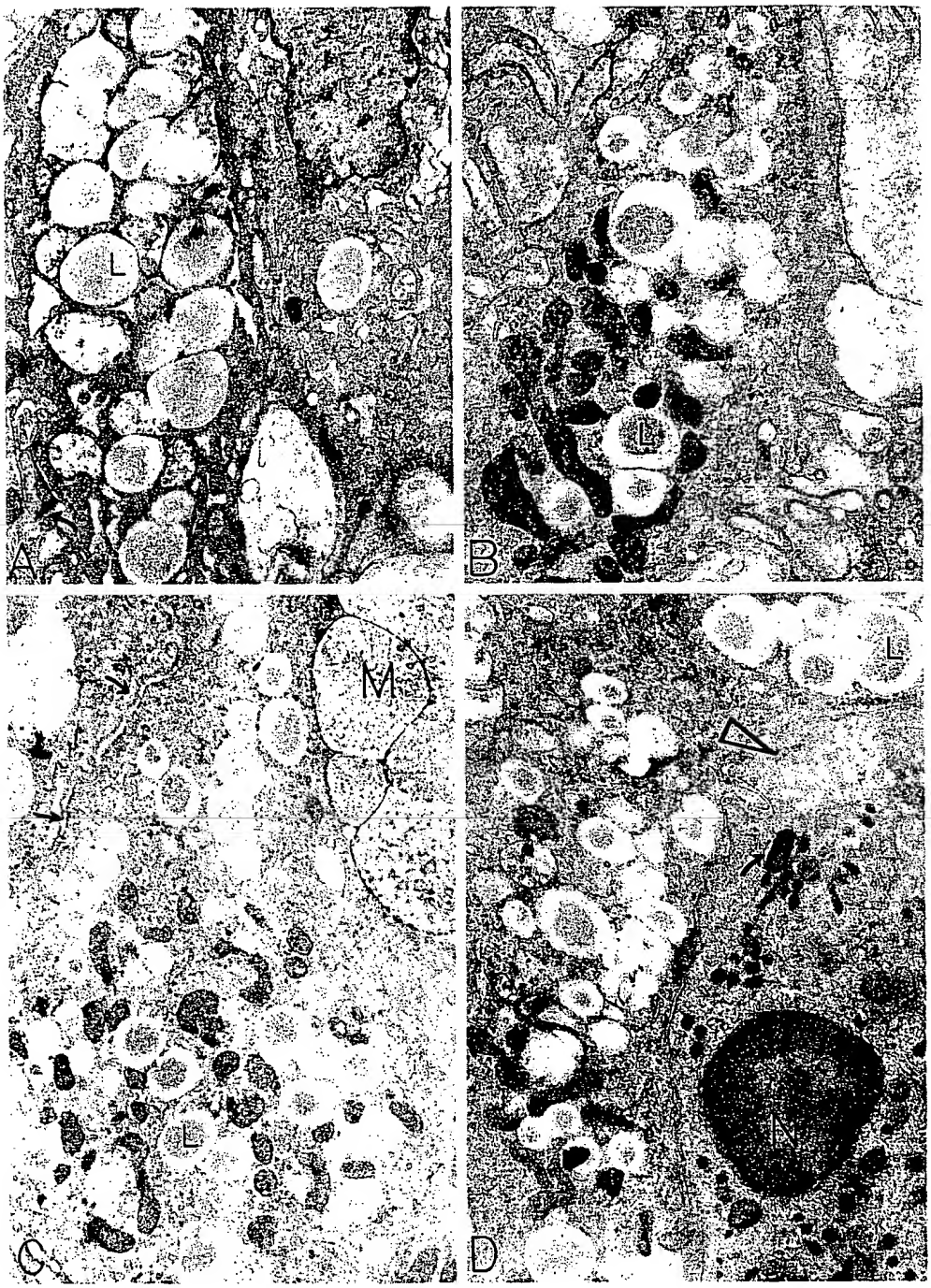

Fig. 5. Absorptive epithelial cells contain numerous nonmembrane-bound, nearly electron-lucent lipid (L) bodies, which are labeled for TNF-α (A, D). The vesicle-rich cytoplasmic area (open arrowhead) of an interepithelial cell neutrophil (N) in (D) also contains TNF-α. A phagocytosed bacterium (arrow) is seen in the neutrophil, adjacent to the TNF-α–labeled vesicles. Nonimmune rabbit serum (B) and TNF-α absorption (C) controls are negative. Intercellular junctions (A, arrow) and basal lamina (C, arrows) define these lipid-laden epithelial cells. Mucous (M) granules are noted in an adjacent mucus-containing epithelial cell (C). (A) ×10,500; (B) ×18,000; (C, D) ×12,500 (before 25% reduction).

antibody with rhTNF-α resulted in a 79% reduction of lipid body label (Fig. 3C) ($P = .038$) and an 84% reduction of the extracellular space label around mast cells ($P = .0031$).

The relative density of TNF-α in lipid bodies of activated mast cells compared to the lipid bodies of other cells is shown in Table 3. While these structures were labeled in mast cells, their rank order of label in cells in the morphometric analysis was fourth, that is, after the lipid bodies of fibroblasts, eosinophils, and macrophages. For granule or phagolysosome rank order, mast cell granules were last of the four cells examined, ranking beneath eosinophils, macrophages, and Paneth cells. For total cell rank order, mast cells were third, between eosinophils (second) and macrophages (fourth).

Macrophages

Tissue macrophages were distinguished by numerous phagolysosomes (containing heterogeneous contents) and nonmembrane-bound, poorly osmiophilic cytoplasmic lipid bodies (structures known to contain arachidonic acid and cyclooxygenase [63, 65]) (**Fig. 4**). Varying numbers of primary lysosomes, endocytotic vesicles and vacuoles, and nondilated cisterns bound by rough endoplasmic reticulum were also features of these cells.

Macrophage intracellular organelles contained TNF-α. The labeled structures included lipid bodies and secondary lysosomes (Fig. 4 and Table 2). Primary lysosomes were not labeled above background. We noted that phagolysosomes containing eosinophil granules (released from dying eosinophils) had particularly strong gold label

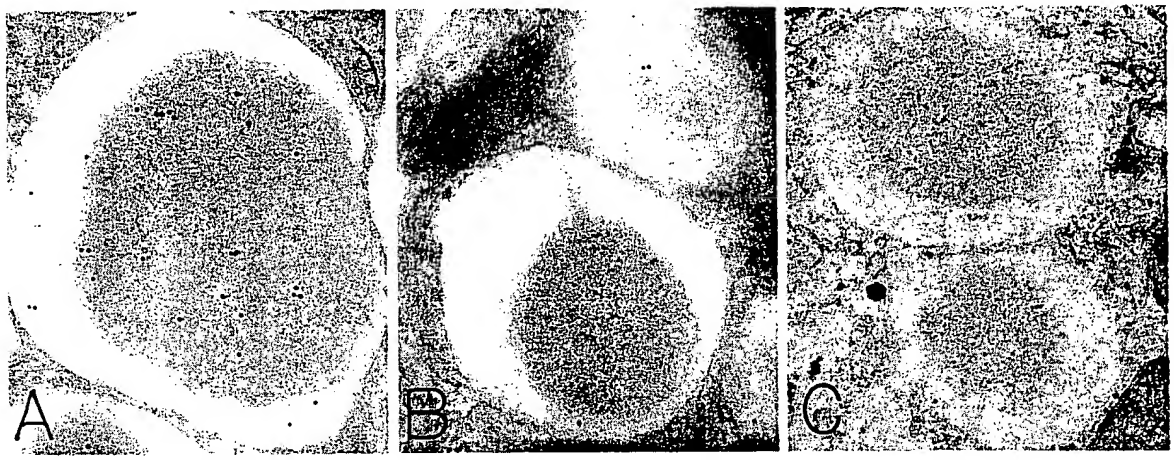

Fig. 6. Higher magnification views of lipid bodies in absorptive epithelial cells of the colon show extensive labeling for TNF-α (A) that is abrogated when nonimmune rabbit serum (B) or TNF-α–absorbed specific antibody (C) was substituted for the primary antibody to TNF-α. (A) ×37,000; (B, C) ×44,500.

for the presence of TNF-α (data not shown).

Specificity controls for the immunogold localization of TNF-α showed that lipid bodies ($P < .0001$ vs. anti-TNF-α values) and phagolysosomes ($P < .001$ vs. anti-TNF-α values) were not labeled when nonimmune rabbit serum was substituted for the specific primary antibody to TNF-α (Fig. 4B). Prior absorption of the specific antibody with rhTNF resulted in a 94% reduction of lipid body label (Table 2) ($P = .0002$) and a 79% reduction of phagolysosome label (Fig. 4D; Table 2) ($P = .001$).

TNF-α was present in the extracellular space around macrophages, a value 27.5-fold higher than the Epon background (Table 2). This TNF-α–containing extracellular space showed abrogation of 81% of the label in the absorption control ($P < .01$) and did not stain significantly when nonimmune rabbit serum was substituted for the primary specific antibody to TNF-α ($P < .01$ vs. anti-TNF-α value).

Table 3 shows the relative density of TNF-α in macrophages, for cells, extracellular space, and organelles. Macrophages ranked fourth of the six cell types analyzed. Macrophage lipid bodies contained a higher density of label than the phagolysosomes or the extracellular space near macrophages. The relative labeling of macrophage lipid bodies ranked third of the five cells analyzed for lipid body label. When the relative labeling of membrane-bound granules or phagolysosomes of the cell types containing them was compared, macrophage phagolysosomes ranked second to eosinophil granules. When

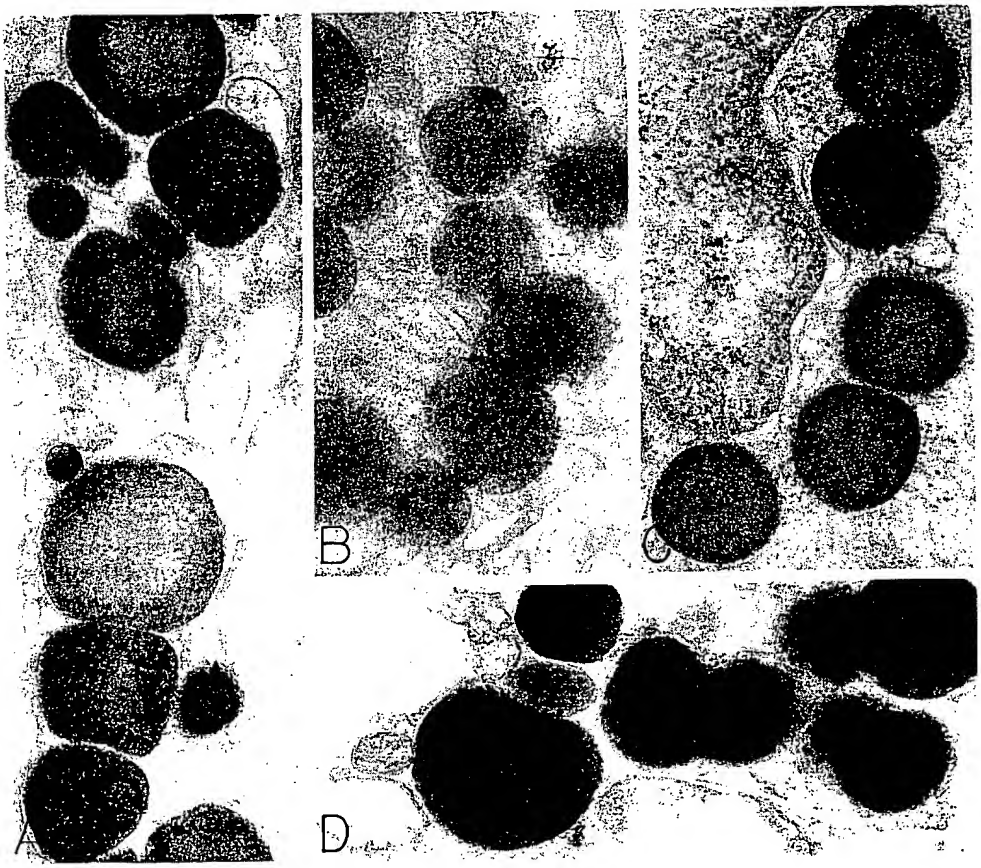

Fig. 7. Fibroblasts show TNF-α-labeled cytoplasmic lipid bodies (A, B) that do not stain in TNF-α absorption (C) and nonimmune rabbit serum (D) controls. (A) ×27,000; (B) ×31,000; (C, D) ×52,000 (before 25% reduction).

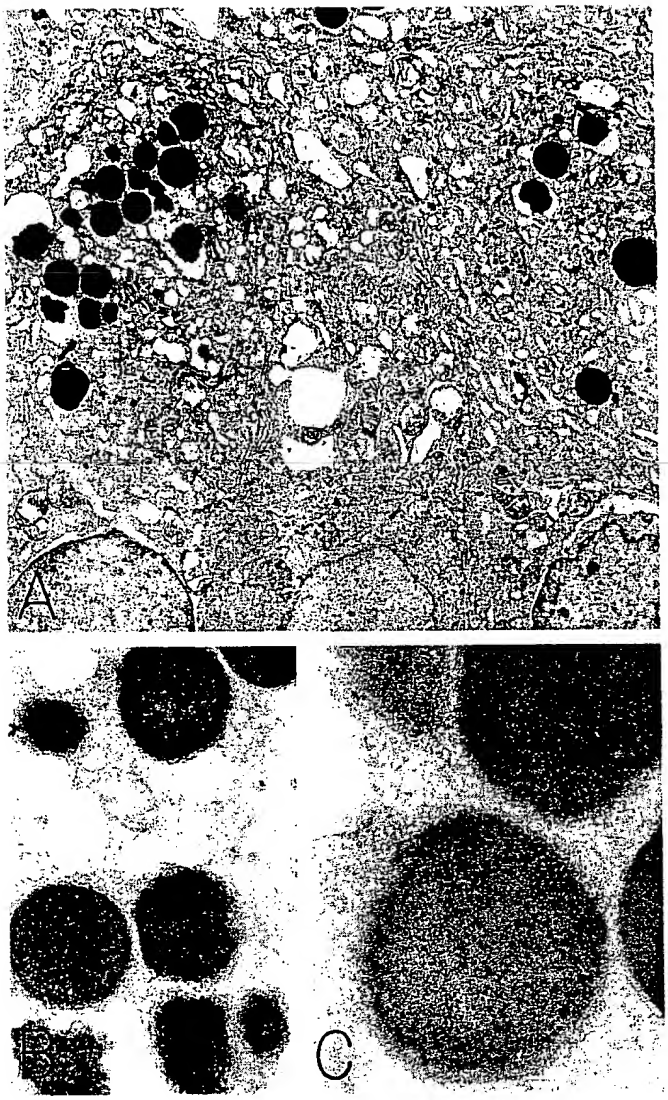

Fig. 8. Paneth cells in the epithelial layer of this colonic biopsy (which contained metaplastic small intestinal–type epithelium) show TNF-α in their secretory granules but not elsewhere. Labeled Paneth cell granules from the cell in (A) are shown at higher magnifications in (B) and (C). (A) ×5,000; (B) ×17,000; (C) ×32,500 (before 2% reduction).

the rank order of macrophage organelles was related to those of all cells analyzed, macrophage phagolysosomes ranked low, just above Paneth cell granules and below lipid bodies of all cells, including macrophages.

Colonic absorptive epithelial cells

Colonic biopsies from one patient (case 5) showed extraordinary numbers of cytoplasmic lipid bodies in the cytoplasm of epithelial cells (**Figs. 5 and 6**), as has been reported for intestinal epithelium in Crohn's disease [68], Cronkite-Canada syndrome [69], and celiac disease [70]. Generally, the lipid bodies were present in absorptive epithelial cells; smaller numbers were noted in mucous epithelial cells. The epithelial cells displaying lipid bodies did not show cellular morphological criteria for injury; some of these cells also contained phagolysosomes with heterogeneous contents. The lipid bodies in epithelial cells were not membrane bound and were poorly osmiophilic. Some were relatively osmiophilic centrally, with diminished density around these centers (Figs. 5 and 6).

TNF-α was uniformly present in lipid bodies of epithelial cells, showing a 180-fold increase over Epon background label (Figs. 5 and 6; Table 3). Phagolysosomes in epithelial cells also contained TNF-α (data not shown). The absorption control revealed 96% reduction of specific label for TNF-α in lipid bodies ($P < .0007$) (Figs. 5C and 6C); nonimmune rabbit serum did not label these structures ($P < .0124$ vs. anti-TNF-α values) (Figs. 5B and 6B).

While only one patient showed epithelial lipid bodies in quantity, we were able to determine the labeling density for 48 lipid bodies in eight different epithelial cells (18 gold/μm^2) and to compare this labeling density to that of similar structures in other cells in the samples. This analysis showed that while, overall, epithelial cells ranked fifth among the six positive cell types, the labeling density of their lipid bodies (18/μm^2) ranked above that of secretory granules in Paneth cells (10/μm^2), which were also present in the epithelial layer. However, the epithelial lipid bodies had the lowest relative labeling density of lipid bodies in the five cell types that were evaluated morphometrically.

Fibroblasts

Fibroblasts in these biopsies generally contained markedly dilated cisterns of the rough endoplasmic reticulum. These spindle-shaped cells also contained nonmembrane-bound osmiophilic lipid bodies—structures known to contain cyclooxygenase [54, 56] (**Fig. 7**). The fibroblast lipid bodies contained TNF-α (Fig. 7 and Table 2), which was reduced by 86% when rhTNF-α–absorbed primary antibody was used ($P < .001$) (Fig. 7C). When nonimmune rabbit serum was substituted for specific antibody, label was absent ($P = .0002$ vs. anti-TNF-α value) (Fig. 7D). The relative density of this label in fibroblast lipid bodies (44/μm^2) was the highest of all lipid bodies studied, and the rank order of labeling density for fibroblasts exceeded that of all cells analyzed (Table 3).

Neutrophils

Rare neutrophils were present in the epithelial cell layer and/or in the underlying lamina propria of the colonic mucosa in all cases. As has been generally reported for Crohn's disease [47], epithelia were less frequently invaded by neutrophils than by eosinophils; focal neutrophil collections in the lamina propria were rarely evident. A few of the neutrophils present in the Crohn's disease samples had small numbers of lipid bodies. A few neutrophils contained TNF-α (data not shown). This label was not present in cytoplasmic granules. Rather, it was associated with the membranes of Golgi structures and cytoplasmic vesicles (data not shown). Some neutrophils showed phagolysosome-related TNF-α, and some poorly osmiophilic lipid bodies were labeled as well (data not shown).

Paneth cells

The colonic biopsies of several cases showed Paneth cell metaplasia. These small intestinal-type epithelial cells were plentiful in case 2—postileocolectomy at the colonic biopsy site. Other cases with Paneth cells included cases 1 and 5. Paneth cells are known to contain TNF-α mRNA [26, 29], and these cells served as a good positive control for this study.

The large secretory granules (both immature and mature) in Paneth cells were regularly positive for TNF-α (10 gold/μm^2) (**Figs. 8 and 9A, D**). The specific absorption

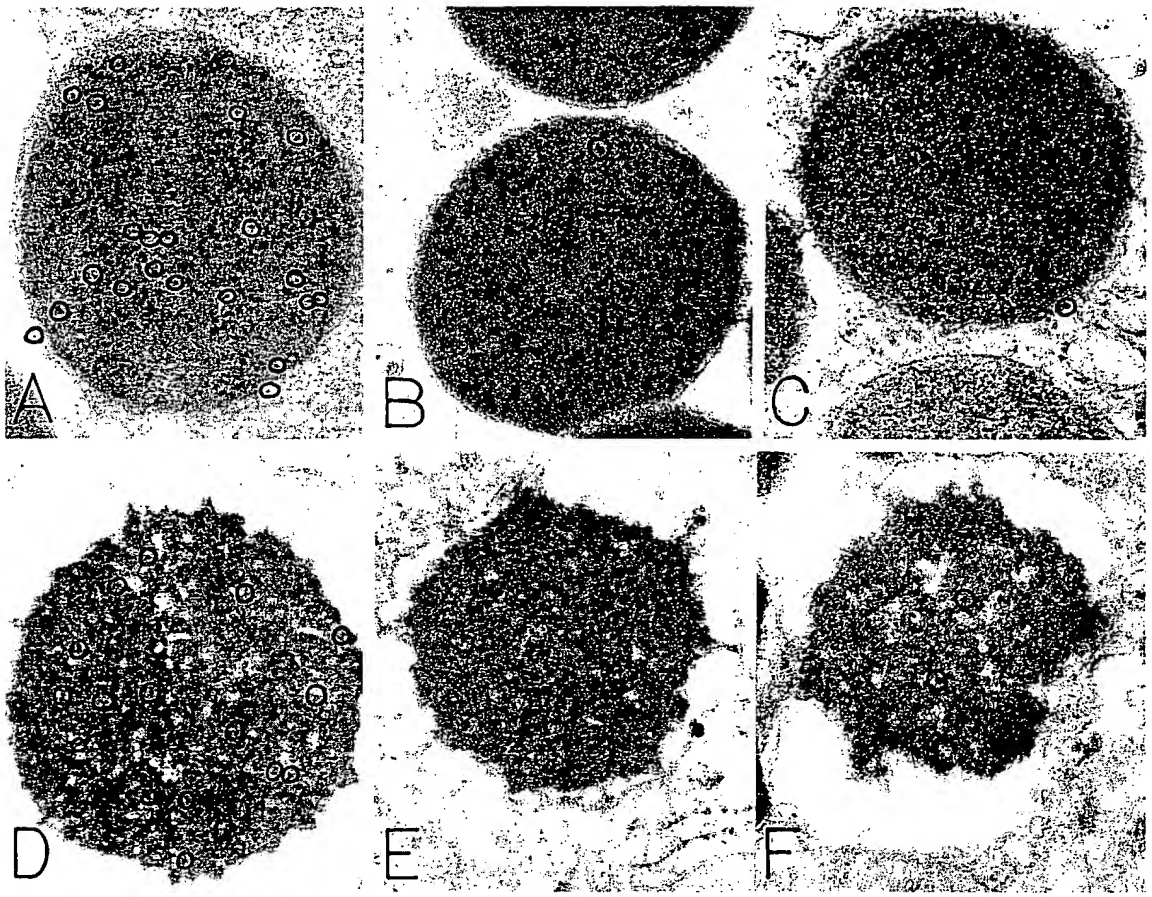

Fig. 9. Higher magnification micrographs of mature (A–C) and immature (D–F) Paneth cell granules show label for TNF-α (A, D) that is absent in nonimmune rabbit serum (B, E) controls and reduced in TNF-α absorption (C, F) controls (circles enclose a representative 10-nm gold particle in each panel). (A) ×35,000; (B) ×50,000; (C) ×43,000; (D) ×27,000; (E) ×55,000; (F) ×51,500.

control reduced this label by 92% ($P < .0101$) (Fig. 9C, F), and nonimmune rabbit serum did not label the granules in Paneth cells ($P < .001$ vs. anti-TNF-α value) (Table 2 and Fig. 9B, E).

The relative labeling density for Paneth cells ranked last of the six cell types analyzed, and their secretory granules contained considerably less TNF-α ($10/\mu m^2$) compared to eosinophil secretory granules ($34/\mu m^2$). As in previous reports [71, 72], Paneth cells did not have lipid bodies.

DISCUSSION

We used an ultrastructural immunogold morphologic and morphometric analysis to identify the cellular, extracellular, and subcellular sites of TNF-α in colonic biopsy tissues obtained from patients with Crohn's disease—a chronic inflammatory disorder of unknown etiology. Using similar approaches, we previously localized TNF-α to the secondary granule matrix compartment of peripheral blood eosinophils of patients with hypereosinophilic syndrome (HES) [40] and the secretory granules of purified rat peritoneal mast cells [44], a site that is depleted of TNF-α early in the release reaction stimulated by 48/80 (W.J. Beil et al., unpublished data). However, this study represents our first effort to identify the tissue localization(s) of TNF-α in vivo in human disease. We chose to examine the lesions of Crohn's disease because of our extensive background in the ultrastructural pathology of this chronic inflammatory disorder (reviewed in refs. 47, 68) and because it is well known that a prominent host inflammatory response is often present by ultrastructure in biopsies from Crohn's disease patients even when the biopsy sites appear uninvolved by endoscopic and light microscopic evaluations [47]. Indeed, ultrastructural approaches, whether to detect evidence of inflammation or to identify sources of TNF-α or other cytokines during this disease process, may represent a more sensitive method for characterizing the inflammation associated with this disorder than any of the more commonly used biochemical or light microscopic approaches for evaluating tissue, blood, or stool samples [11–14, 16–18, 23, 24].

We identified TNF-α in tissue eosinophils, macrophages, mast cells, and neutrophils, as well as in fibroblasts, absorptive epithelial cells, and Paneth cells. Subcellular locations of TNF-α were identified in lipid bodies (the density of labeling of lipid bodies in these cells was fibroblasts > eosinophils > macrophages > mast cells > colonic epithelial cells, neutrophils), secretory granules (eosinophils > Paneth cells), phagolysosomes (macrophages, colonic epithelial cells), Golgi structures, and cytoplasmic vesicular membranes (neutrophils). Extracellular spaces within 2 μm of three inflammatory cell lineages also contained TNF-α at labeling densities that were significantly greater than background values in the same sections (eosinophils > mast cells > macrophages). Substituting nonimmune rabbit serum for the specific primary antibody did not result in any significant gold labeling of these cells, subcellular sites, or extracellular spaces, and prior absorption of the specific

antibody to TNF-α with rhTNF-α resulted in significant (79–96%) reductions of gold labeling, compared with sham-absorbed positive controls.

Thus, the increased resolution afforded by immunogold electron microscopy enabled us to identify TNF-α in subcellular sites of at least seven cell lineages present in these Crohn's disease colonic biopsies. We also generated evidence for the release of TNF-α into the extracellular microenvironment surrounding three inflammatory cell lineages, eosinophils, mast cells, and macrophages. Ours are the first ultrastructural studies to characterize the distribution of TNF-α in Crohn's disease tissues. Our findings should aid in future efforts to understand the role of TNF-α in the pathogenesis of Crohn's disease.

The cells that contain TNF-α in Crohn's disease colonic tissues are of interest. Eosinophils are a constant feature of the cellular infiltrate in gut tissues in Crohn's disease, and the ultrastructural morphology of eosinophils in these sites indicates that they are activated (reviewed in refs. 47, 52, 68). For example, eosinophils in these tissues often have increased lipid bodies and altered secondary granules, indicating losses of granule matrix and/or crystalline core compartments associated with piecemeal degranulation (PMD)—a secretory process effected by vesicular transport of granule materials [60, 61]. In this study, we did not find evidence for regulated secretion by extrusion of membrane-free granules [59], a result consistent with our previous studies of Crohn's disease tissues (reviewed in ref. 47). Previously, we localized TNF-α to the secondary granules of HES peripheral blood eosinophils, using ultrastructural methodology similar to that used here [40]. Additionally, we have shown that HES eosinophils synthesize and secrete TNF-α [39]. Others have localized TNF-α transcripts to eosinophils in necrotizing enterocolitis tissues [26]. The label density of TNF-α around eosinophils in Crohn's disease colonic tissues was higher than that around either mast cells or macrophages—the three cells examined in this way in the present study. Also, the relative label density of eosinophils was the highest of any of the TNF-α–positive inflammatory cells in these biopsies. Of the eosinophil subcellular organelles that contained TNF-α, secretory granules and lipid bodies had similar label densities, values that were ~50% higher than in the immediate microenvironment of eosinophils. Eosinophil lipid bodies are known to play an important role in eicosanoid metabolism [52–58]. Thus, eosinophils represent a major potential source of TNF-α in colonic Crohn's disease.

Mast cells are also increased in number in Crohn's disease tissues, and their ultrastructural morphology reveals that they are activated as well (reviewed in refs. 47, 68). Activation of gut mast cells in Crohn's disease is primarily reflected by increased numbers of lipid bodies in these cells and losses from their electron-dense secretory granules that are characteristic of PMD. Some mast cells are hypogranular, reflecting immaturity and/or granule reduction from prior secretory events. (Quantitation of immunogold TNF-α label for the granule compartment of mast cells in this study excluded partially, or completely, empty granule containers and reflects solely that of completely full granules for comparison with the lipid body compartment.) Mast cells are now recognized as a potentially major source of TNF-α, since it has been reported that the mast cells of several species can synthesize, contain, and secrete TNF-α [41–44] (reviewed in refs. 45, 46). We recently applied an ultrastructural immunogold analysis, similar to that reported here, to show that rat peritoneal mast cells stored TNF-α in their large, electron-dense secretory granules [44] and secreted this proinflammatory cytokine from this compartment when stimulated with compound 48/80 (W.J. Beil et al., unpublished data). Others have shown that human skin mast cell granules contain TNF-α [42].

However, in the current study, the cytoplasmic granules of mast cells in the Crohn's disease colonic biopsies did not contain TNF-α gold label levels that exceeded background levels (0.1 gold particle/μm^2). On the other hand, we did find a high label for TNF-α in lipid bodies in the gut mast cells (see later). These structures are regularly absent from the two mast cell populations for which ultrastructural granule localization data exist—rat mast cells [44] (W.J. Beil, unpublished data) and human skin mast cells [42]—but are a regular feature of human gut mast cells (reviewed in ref. 62). The relative label density of TNF-α for mast cell lipid bodies was third in rank order of lipid bodies in inflammatory cells in colonic Crohn's disease (eosinophil > macrophage > mast cell). TNF-α was also present in the immediate microenvironment of mast cells. Lack of label in the granule compartment may reflect prior secretion with reduction of granule label to levels not detectable by the techniques used in this study. Alternatively, lipid bodies may represent the major TNF-α storage site in human gut mast cells. In any event, our findings indicate that mast cells represent a major potential source of TNF-α in colonic Crohn's disease.

Macrophages are plentiful in Crohn's disease tissues, and many of these "professional phagocytes" contain phagolysosomes with heterogeneous contents (reviewed in refs. 47, 68). Macrophages are also secretory cells and, when activated, are generally considered to be a major producer of TNF-α [1–3]. Some studies have identified TNF-α mRNA and protein in tissue histiocytes in inflammatory bowel disease [14] and necrotizing enterocolitis [26], but the macrophage contribution of TNF-α relative to other sources is not known. Although the macrophages we studied in colonic Crohn's disease contained TNF-α, the relative labeling density of these cells was third of the inflammatory cells that we quantitated (i.e., eosinophil > mast cell > macrophage). The microenvironment around macrophages also contained TNF-α, in the same relative density rank order compared to eosinophils and mast cells.

As in eosinophils and mast cells, macrophage lipid bodies contained the highest amount of TNF-α. The relative label density analysis showed that lipid bodies contained twice the TNF-α label compared to the other labeled subcellular compartment, phagolysosomes. Primary lysosomes were not labeled in macrophages. Since a number of macrophage phagolysosomes contained eosinophil granules that had spilled into interstitial tissues from dying cells, much of the TNF-α label in macrophage phagolysosomes could have originated from this source. Alternatively and/or additionally, TNF-α could be secreted into phagolysosomes from the highly labeled macrophage lipid body compartment. We have observed such lipid body intracellular traffic patterns in phagocytosing murine peritoneal macrophages [63] and human alveolar macrophages [63] and skin histiocytes (reviewed in ref. 52). It is possible that there is an important intracellular site of action of TNF-α in the phagocytic, lipid body–rich macrophages of colonic Crohn's disease.

Neutrophils are not a frequent infiltrating cell type in bowel tissues in Crohn's disease, in contrast to ulcerative

colitis (reviewed in ref. 47). Despite the small sample of infiltrating neutrophils in the colonic biopsies we studied, some contained TNF-α, primarily in Golgi and cytoplasmic vesicular membranes. Secretory granules were often reduced in number; these structures did not contain label for TNF-α. Although lipid bodies were infrequent in this population of activated neutrophils, some of these structures contained TNF-α immunoreactivity. In other circumstances, we have documented the rapid, protein kinase C–dependent formation of neutrophil lipid bodies in appropriately stimulated cells [73]. Also, tissue neutrophils laden with lipid bodies in vivo have been recorded [74]. Neutrophil lipid bodies contain arachidonic acid, assayed biochemically and with ultrastructural autoradiography [73, 74]. Other investigators have shown that peripheral blood neutrophils synthesize and secrete TNF-α [36–38]. Thus, while numbers of neutrophils were small in colonic Crohn's disease samples, when present they contained TNF-α.

Fibroblasts in our specimens were large, elongated cells of typical ultrastructural morphology. Morphological evidence of their activation included large amounts of synthetic machinery (Golgi structures and dilated cisterns of rough endoplasmic reticulum) and large, cytoplasmic, osmiophilic lipid bodies [56]. Fibroblasts had the greatest relative density of TNF-α label in comparison to the other six cell types we analyzed in colonic Crohn's disease. These resident mesenchymal cells contained all of their TNF-α in lipid bodies, structures with the highest label density for all subcellular organelles that we quantified in this study. TNF-α is known to have myriad actions on fibroblasts [75–77]; a standard in vitro assay for TNF-α makes use of L cells—a transformed fibroblast cell line [27]. Moreover, L cells can be selected for TNF-α resistance by repetitive exposure to this cytokine, and the resistant cells acquire the ability to produce TNF-α [27]. Our work shows that fibroblasts in colonic Crohn's disease contain high concentrations of TNF-α in their expanded lipid body compartment. Whether this location is the target site of an uptake mechanism from other TNF-α–producing cells in the microenvironment or represents synthesis de novo is not yet known. Fibroblast lipid bodies are sites of eicosanoid metabolism, however, containing a variety of enzymes key to the metabolism of arachidonic acid [54, 56, 58].

Epithelial absorptive cells in colonic Crohn's disease contained TNF-α in numerous large, poorly osmiophilic lipid bodies, as well as in cytoplasmic phagolysosomes. These structures are known to be increased in ileal tissues in Crohn's disease (reviewed in refs. 47, 68), and lipid bodies have been recorded in the epithelia of intestinal tissues in other diseases [69, 70]. The epithelial cells that contained TNF-α showed no morphologic evidence of cell injury. They were well preserved, were connected by lateral surface junctions, and were bound on their basal surfaces by basal lamina. The TNF-α label density for colonic epithelial cells ranked fifth of the six cell types we quantified, and the TNF-α label density of these epithelial cell lipid bodies ranked last of the lipid bodies in five cell types for which quantitative data were obtained. Despite this rank order, the presence of this TNF-α–positive organellar compartment in the surface-lining epithelial cells could indicate that TNF-α of epithelial cell origin might influence the barrier, secretory, and absorption functions of these epithelia [72, 78]. The literature is replete with examples of TNF-α– or TNF-α mRNA–containing epithelial cells. These include alveolar type II pneumocytes in cryptogenic fibrosing alveolitis [30], glandular epithelial cells of the uterus [31], thyroid epithelial cells [32], renal tubular epithelial cells [34], hepatic epithelial cells in viral hepatitis [33], and psoriatic skin epithelium [35]. None of these studies made use of ultrastructural immunolocalization technology to delineate TNF-α–positive subcellular sites. We clearly show, in colonic Crohn's disease, that the absorptive epithelial cells have TNF-α in at least two subcellular sites—lipid bodies and phagolysosomes.

Paneth cells are classical secretory epithelial cells that contain large numbers of membrane-bound secretory granules [71, 72]. They are found in the ileum, increase in number in this location in Crohn's disease, and develop de novo or by metaplasia in colonic sites in patients with inflammatory bowel disease [71, 72]. Neither ileal nor colonic Paneth cells in humans contain lipid bodies [71, 72]. Paneth cells have been shown to contain TNF-α transcripts; they are considered to be the major source of TNF-α in normal bowel, and they have been shown to increase dramatically in TNF-α expression in necrotizing enterocolitis [26]. Despite identifying the presence of TNF-α mRNA in Paneth cells, Tan et al. [26] did not detect TNF-α protein in Paneth cells in human tissue by immunohistochemistry [26]. Others, using normal mouse small intestine, also detected TNF-α mRNA in Paneth cells but could not detect TNF-α by immunohistochemistry or Western blotting [29]. However, neither of these studies made use of the increased sensitivity for subcellular localizations that is provided by ultrastructural immunogold methods analogous to those used in our study. With these techniques, we found TNF-α in mature and immature secretory granules of Paneth cells in colonic Crohn's disease. The rank order of label density/μm^2 for Paneth cells was sixth of the six cell lineages quantified, and the label density for their secretory granules ranked last of all cellular organelles quantified, except for the total absence of TNF-α in macrophage lysosomes and mast cell granules. Thus, it is clear from our study that Paneth cells do contain TNF-α in colonic Crohn's disease, but they are only one of the cell types (among many) that do so. For example, relative density of TNF-α label in the secretory granules of eosinophils was threefold greater than that of Paneth cell secretory granules.

Our immunogold studies of subcellular sites of TNF-α in colonic Crohn's disease showed that a cellular organelle, the lipid body, constituted a major site of immunoreactivity for this proinflammatory cytokine in fibroblasts, eosinophils, macrophages, mast cells, colonic epithelial absorptive cells, and neutrophils. Lipid bodies are cellular cytoplasmic organelles that greatly increase in number during inflammatory processes [47, 52–58, 62–70, 73, 74]. Lipid bodies have been shown to play a key role in eicosanoid metabolism and are known to be repositories of arachidonyl phospholipids, as well as prostaglandin endoperoxide synthase (cyclooxygenase) and 5-lipoxygenase [52–58, 63–65, 73, 74, 79]—enzymes necessary for prostaglandin, thromboxane, and leukotriene synthesis. We now show that lipid bodies may also participate in TNF-α metabolism in colonic Crohn's disease. Since evidence is accumulating that arachidonic acid and/or its metabolites are involved with TNF-α synthesis and function(s) (and vice versa) [4–10, 80–88], our ability to place eicosanoid and TNF-α metabolic events in the same, previously unrecognized subcellular site—the lipid body—should facilitate future efforts to understand the importance of these organelles in health and disease.

ACKNOWLEDGMENTS

Supported by USPHS grants AI-33372, AI-22571, AI-31982, AI-20241, and DK-34854. We thank Peter K. Gardner for editorial assistance in the preparation of the manuscript.

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ORIGINALARBEIT

Ernährung und Morbus Crohn – Ein ätiologischer Faktor?

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Zusammenfassung

Epidemiologische Daten weisen auf eine ätiologische Bedeutung exogener Noxen beim Morbus Crohn. In mehreren Studien wurde der Einfluß von Ernährungsfaktoren auf Krankheitsentstehung oder Rezidivverhütung überprüft. Die Ergebnisse sind zum Teil widersprüchlich, die angewandten Methoden vielfach diskussionswürdig und die untersuchten Patientengruppen oft klein und nur eingeschränkt untereinander oder mit den Kontrollkollektiven vergleichbar. Ein schlüssiger Beweis für die ätiologische Bedeutung der Ernährung bei diesem Krankheitsbild steht noch aus. Somit gibt es keine spezifische »Crohn-Diät«. Die möglichst vielseitige Ernährung der Patienten sollte den kalorischen Bedarf decken, Mangelzustände ausgleichen, krankheitsbedingte Komplikationen wie Malabsorptionssyndrome oder Stenosen und individuelle Unverträglichkeiten berücksichtigen.

Summary

(Nutrition and Crohn's disease – a factor in etiology?)

Epidemiological data indicate that exogenous noxes are important in the etiology of nonspecific inflammatory bowel disease. In several studies the influence of nutritional factors in the pathogenesis of Crohn's disease was investigated. The conflicting results, the inappropriate methods of investigation, the limited number of patients, and differences between patients and controls in many of these studies require a careful interpretation. Evidence for an etiological significance of nutrition in the development of Crohn's disease is still missing. Therefore, a specific diet for these patients does not exist. The physician should recommend a balanced diet which considers the needs in energy supply, corrects preexisting deficiencies, and is adapted to subjective intolerances and to disease related complications such as malabsorption or partial intestinal obstruction.

Auch über 50 Jahre nach der Erstbeschreibung des Morbus Crohn als Krankheitsentität ist die Ätiologie der Erkrankung noch unbekannt. Eine Vielzahl von unterschiedlichen Faktoren wurde für die Entstehung dieses Krankheitsbildes verantwortlich gemacht. Das diskutierte Spektrum pathogenetischer Mechanismen beinhaltet bakterielle, autoimmunologische, allergische, genetische, nutritive und toxische Ursachen (1). Epidemiologische Studien, die eine steigende Inzidenz in industrialisierten Ländern und eine im Vergleich zu ländlichen Regionen höhere Prävalenz in städtischen Gebieten (2, 3) feststellten, weisen auf die Bedeutung exogener Faktoren hin. Die regional unterschiedliche Häufigkeit des Morbus Crohn geht parallel zu veränderten Ernährungsgewohnheiten in der Bevölkerung mit Bevorzugung schlackenarmer und zuckerreicher Kost. Daher wurde in verschiedenen Untersuchungen der Stellenwert der Ernährung bei der Entstehung bzw. Rezidivverhütung chronisch entzündlicher Darmerkrankungen überprüft.

Im folgenden werden die Ergebnisse dieser Untersuchung aufgezeigt und hinsichtlich der Relevanz für die Betreuung Crohn-Kranker diskutiert.

Eingesetzte Untersuchungsmethoden

In 12 Studien wurden die Ernährungsgewohnheiten der Patienten zum Zeitpunkt des meist Monate bis Jahre zurückliegenden Krankheitsbeginns retrospektiv mit Hilfe von Fragebögen oder Interviews erfaßt (4–15). Zwei Untersucher (11–16) werteten über längere Zeit geführte Ernährungsprotokolle aus. *Heckers* (17) bestimmte die im subkutanen Fett gespeicherten Fettsäuren, die der Körper selbst weder synthetisieren noch abbauen kann und die somit ein Maß für die Aufnahme dieser Substanzen mit der Nahrung darstellen. Sechs Studien (18–23) überprüften prospektiv den Einfluß einer diätetischen Intervention auf den Krankheitsverlauf. Neben diesen mit unterschiedlichen Kontrollkollektiven durchgeführten Untersuchungen wurden zwei Erfahrungsberichte (24, 25) über die Bedeutung einer Diät bei der Therapie des Morbus Crohn publiziert.

Wenn auch ein allgemein akzeptiertes Tiermodell für die chronisch entzündlichen Darmerkrankungen noch nicht existiert (26), so wurde dennoch die ätiologische Bedeutung verschiedener Nahrungsbestandteile tierexperimentell überprüft.

Zuckerkonsum

Acht Studien (6, 7, 9–13, 15), die insgesamt 516 Patienten mit Morbus Crohn erfaßten, stellten einheitlich einen hohen Zuckerkonsum fest; bei sieben Untersuchungen waren erhebliche Unterschiede im Vergleich zu einem Kontrollkollektiv feststellbar. Nach Krankheitsbeginn war der Zuckeranteil der Ernährung in zwei Studien (7, 11) weiter angestiegen, während zwei andere Studien (12, 13) einen Rückgang des Zuckerkonsums im Krankheitsverlauf feststellten. Bei Verwendung eines Ernährungsprotokolls über eine Woche wies *Kasper* (16) bei 35% der Patienten mit Morbus Crohn einen sehr hohen Zuckerverbrauch nach. *Mayberry* (11) konnte in einer ähnlich konzipierten Studie keinen signifikanten Unterschied zwischen Patienten und einer Kontrollgruppe nachweisen. Bei zwei prospektiven Studien (18, 22) wurde der Stellenwert einer zuckerarmen Diät zur Rezidivprophylaxe überprüft; ein positiver Effekt der diätetischen Intervention ließ sich jedoch nicht feststellen (Tab. 1).

Kohlenhydratkonsum

Drei englische Arbeitsgruppen befaßten sich mit dem Kohlenhydratanteil in der Ernährung von Crohn-Patienten. Während *Ja-*

Manuskript eingegangen: 16.01.1989
In vorliegender Form angenommen: 24.04.1989

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mes (8) bei 34 Patienten im Vergleich zur Kontrollgruppe einen höheren Verbrauch an Cornflakes und Weizenmehlprodukten zum Frühstück feststellte, haben *Archer* und *Rawcliffe* (4, 14) diesen Befund an größeren Patientenzahlen nicht reproduzieren können. *Heaton* u. Mitarb. (19) wiesen bei 32 Patienten unter einer kohlenhydratarmen und faserreichen Kost im Vergleich zu einer historischen Kontrollgruppe eine Verringerung der Krankenhausaufenthalte und chirurgischen Eingriffe nach. *Lutz* (24) schilderte seine Erfahrungen an 103 Patienten, die unter kohlenhydratarmer Ernährung in 88% der Fälle eine Besserung zeigten; Kriterien für die Beurteilung des Krankheitsverlaufs oder ein Vergleichskollektiv führte er nicht an (Tab. 2).

Tab. 1: Studien zum Zuckerkonsum bei Morbus Crohn

Patienten	Kontrollen	Zuckerkonsum	Methode	Referenz
103		+	Fragebogen	6
30	30	+ ($p < 0{,}05$)	Interview	7
104	153	+ ($p < 0{,}005$)	Fragebogen	9
63	63	+ ($p < 0{,}02$)	Fragebogen	10
32	32	+ ($p < 0{,}01$)	Fragebogen	11
120	120	+ ($p < 0{,}01$)	Fragebogen	12
34	34	+ ($p < 0{,}01$)	Interview	13
30	30	+ ($p < 0{,}02$)	Fragebogen	15
		in 35% hoch	Protokoll	16
16	16	n.s.	Protokoll	11
10	10	n.s.	Diättherapie	18
190	162	n.s.	Diättherapie	22

Zeichenerklärung:
+ gegenüber der Kontrollgruppe erhöht (Signifikanzgrenze)
n.s. kein signifikanter Unterschied gegenüber der Kontrollgruppe

Tab. 2: Studien zum Kohlenhydratkonsum bei Morbus Crohn

Patienten	Kontrollen	Kohlenhydratkonsum	Methode	Referenz
57	57	n.s.	Interview	4
34	68	+ ($p < 0{,}01$)	Interview	8
100	100	n.s.	Interview	14
32	32	+ +	Diättherapie	19
103		+ +	Diättherapie	24

Zeichenerklärung:
+ gegenüber einer Kontrollgruppe erhöht (Signifikanzgrenze)
+ + positiver Einfluß auf den Krankheitsverlauf
n.s. kein signifikanter Unterschied gegenüber der Kontrollgruppe

Faseranteil in der Ernährung

Zwei retrospektive Studien an kleinen Kollektiven kamen zu divergenten Ergebnissen; während *Thornton* (15) einen im Vergleich zu Gesunden signifikant geringeren Ballaststoffanteil in der Kost von Crohn-Patienten feststellte, fand *Kasper* (16) keinen Unterschied im Fasergehalt der Ernährung zwischen beiden Gruppen. *Heaton* (19) stellte im Vergleich zu einer historischen Kontrolle unter kohlenhydratarmer, faserreicher Kost einen positiven Einfluß der Diät auf den Krankheitsverlauf fest. Zwei prospektive Studien (21, 22), die den Stellenwert des Fasergehaltes der Ernährung zur Rezidivverhütung über einen Zeitraum von mindestens zwei Jahren überprüften, wiesen keinen positiven Einfluß der diätetischen Intervention nach. *Ritchie* (22) beobachtete unter faserreicher Kost sogar eine Zunahme der subjektiven Beschwerden (Tab. 3).

Fettkonsum

Guthy (6) befragte 103 Patienten mit Morbus Crohn zu ihrem Ernährungsverhalten; alle hatten vor Krankheitsbeginn chemisch aufbereitete Fette zu sich genommen. Da solche Fette nicht vom Körper synthetisiert werden können und entsprechend ihrem Anteil in der Nahrung im subkutanen Gewebe gespeichert werden, untersuchte *Heckers* (17) das Fettsäuremuster der Subkutis von 22 Patienten mit Morbus Crohn. Hierbei stellte er bei einer Majorfettsäure (Anteil > 1% an den Gesamtfettsäuren), die aus chemisch aufbereiteten Fettsäuren stammte, einen signifikanten Unterschied im Vergleich zu Kontrollpersonen fest. *Brandes* (5) wies keinen Unterschied im Fettkonsum bei Crohn-Patienten und einem Vergleichskollektiv nach (Tab. 4).

Tab. 3: Studien zum Faseranteil der Ernährung bei Morbus Crohn

Patienten	Kontrollen	Faseranteil	Methode	Referenz
		n.s.	Protokoll	16
30	30	− ($p < 0{,}05$)	Fragebogen	15
36	35	n.s.	Diättherapie	21
190	162	n.s.	Diättherapie	22
32	32	+ +	Diättherapie	19

Zeichenerklärung:
− gegenüber einer Kontrollgruppe erniedrigt (Signifikanzgrenze)
+ + positiver Einfluß auf den Krankheitsverlauf
n.s. kein signifikanter Unterschied gegenüber der Kontrollgruppe

Tab. 4: Studien zum Fettkonsum bei Morbus Crohn

Patienten	Kontrollen	Fettkonsum	Methode	Referenz
103		*	Fragebogen	6
63	63	n.s.	Fragebogen	5
22	22	** ($p < 0{,}05$)	Gewebeanalyse	17

Zeichenerklärung:
* Konsum chemisch aufbereiteter Fette
** Unterschied gegenüber der Kontrollgruppe bei einer Majorfettsäure (Signifikanzgrenze)
n.s. kein signifikanter Unterschied gegenüber der Kontrollgruppe

In einer Mitteilung berichtete *Nagel* (26) über Mukosaveränderungen im Tierversuch unter fettreicher Ernährung. Nach Anlage eines antiperistaltischen Ileumsegmentes wurden Schweine über drei Monate entweder normal oder fettreich (Fettanteil: 60% der Gesamtkalorien) ernährt. Unter der Experimentaldiät wurden ein Mukosaödem, eine Abnahme des Becherzellgehaltes, eine Zottenverkürzung und eine entzündliche Schleimhautinfiltration beobachtet.

Eliminationskost

Der Begriff Eliminationskost wird von einzelnen Arbeitsgruppen unterschiedlich definiert. *Lutz* (24) versteht darunter eine Kost mit einem Kohlenhydratanteil von etwa 15%, die wenig potentiell allergisierende Substanzen enthält. Unter einer derartigen Ernährung beobachtete er bei seinen Patienten einen günstigen Krankheitsverlauf. *Tschaikowski* (25) testete eine allergenarme Ernährung bei 14 Patienten jeweils individuell aus. *Jones* (20) wertete den Krankheitsverlauf von 64 Patienten unter einer nach subjektiver Verträglichkeit ausgewählten Kost aus und stellte eine günstige Wirkung einer derartigen Ernährung fest. In einer randomisierten Studie an 20 Patienten, die sich über sechs Monate faserreich oder mit einer individuellen Elimina-

tionskost ernährten, wiesen Patienten unter der letztgenannten Ernährungsform signifikant weniger Rezidive auf (20). *Schmid* (23) konnte diese Ergebnisse in einer vergleichbaren Studie an 28 Patienten, die über ein Jahr beobachtet wurden, nicht reproduzieren. Hierbei wurde eine Patientengruppe aufgefordert, sich mit einer normalen, subjektiv gut verträglichen Kost zu ernähren, während in einer zweiten Gruppe individuelle Intoleranzen durch systematische Erweiterung einer Basisdiät nach zuvor festgelegtem Plan nachgewiesen wurden. Die beiden Therapiegruppen unterschieden sich weder im Krankheitsverlauf noch in der Zahl und Art der entweder spontan oder im Rahmen des systematischen Kostaufbaus eliminierten Nahrungsmittel.

Tab. 5: Studien zur Eliminationskost bei Morbus Crohn

Patienten	Kontrollen	Eliminationskost	Methode	Referenz
64		+ +	Diättherapie	20
103		+ +	Diättherapie	24
14		+ +	Diättherapie	25
10	10	+ +	Diättherapie	20
14	14	n.s.	Diättherapie	23

Zeichenerklärung:
+ + positiver Einfluß auf den Krankheitsverlauf
n.s. kein signifikanter Unterschied gegenüber der Kontrollgruppe

Nahrungsmittelzusätze

In den siebziger Jahren wurden im Tierversuch Ulzera im proximalen Dickdarm bei Gabe von Carrageninen beobachtet. Diese sulfatierten Polysaccharide werden als Stabilisatoren in der Nahrungsmittelindustrie bei der Herstellung von Süßspeisen verwandt. Eine experimentelle Kolitis trat jedoch nur bei einigen Spezies auf. Auch über 200 Patienten, die Präparate dieser Substanzgruppe zur Therapie von gastroduodenalen Ulzera über einen längeren Zeitraum erhielten, zeigten keine Symptome einer entzündlichen Dickdarmerkrankung (27).

Diskussion

Wenn auch die Assoziation zwischen steigender Inzidenz des Morbus Crohn und den geänderten Lebensgewohnheiten der modernen Industriegesellschaft auf eine ätiologische Bedeutung exogener Faktoren hinzuweisen scheint, so konnten die bisher publizierten Studien keinen Beweis für den Einfluß der Ernährung auf die Krankheitsentstehung erbringen. Die Resultate der Studien, die Ernährungsgewohnheiten vor Krankheitsbeginn retrospektiv zu erfassen versuchten, müssen mit großer Vorsicht interpretiert werden. Vielfach werden die aktuellen Ernährungsgewohnheiten in die Vergangenheit projiziert (4, 7, 11, 28). Wenn nun Unterschiede in der Ernährung von Crohn-Patienten und einem Kontrollkollektiv bestehen, so läßt sich schwer feststellen, ob es sich um ursächliche Faktoren für die Krankheitsentstehung handelt oder ob die Patienten beispielsweise aufgrund einer Obstruktionssymptomatik oder einer Malassimilation auf gut resorbierbare, hochkalorische Nahrungsmittel zurückgegriffen haben. Es bleibt spekulativ, ob bei der in vielen Studien überprüften Rezidiventstehung die gleichen Faktoren eine Rolle spielen wie bei der Krankheitsentstehung. Da durch die chronische Entzündung Leistungen der intestinalen Mukosa wie unspezifische Permeabilität und Resorption verändert sind, können Nahrungsbestandteile Beschwerden hervorrufen oder Noxen darstellen, die bei intakter Schleimhaut keinerlei Auswirkungen auf den Organismus haben. Aber auch die Rezidivverhinderung mit diätetischen Maßnahmen scheint nach den jüngst veröffentlichten Studien nicht möglich zu sein (21–23).

Kleine Fallzahlen, kurze Beobachtungszeiten und fehlende oder nicht wirklich vergleichbare Kontrollgruppen schränken die Aussage einiger Untersuchungen noch weiter ein (6, 17–20, 24, 25, 28, 29). Die Einzelwerte der überprüften Nahrungsbestandteile streuten meist sehr stark, so daß sich die Angaben von Crohn-Patienten und Kontrollpersonen weit überlappten (8, 9, 11, 13, 15–17) und somit die Hinweise auf eine ätiologische Bedeutung der entsprechenden Faktoren nur für einen Teil der Betroffenen galten. *Persson* (28) hält aus diesem Grund die meist angewandten statistischen Verfahren mit Mittelwertvergleichen für ungeeignet.

Was ist nun gesichert in der diätetischen Beeinflussung des Morbus Crohn? Bisher weisen nur die eingangs genannten theoretischen Erwägungen und die Resultate einiger, zum Teil allerdings methodisch zumindest diskussionswürdiger Studien auf eine ätiologische Bedeutung der Ernährung hin. Die für den praktisch tätigen Arzt mindestens genauso wichtige sekundäre Prävention im Sinne einer Rezidivverhütung erscheint nach bisherigen Kenntnissen durch eine diätetische Intervention nicht möglich. Daß eine undifferenziert nach Protokoll empfohlene Ernährung mit faserreicher, zuckerarmer Kost sogar eine Beschwerdezunahme verursachen kann, läßt sich aus der Untersuchung von *Ritchie* (22) entnehmen. In dieser Studie wurden auch Patienten mit intestinalen Stenosen nach Randomisierung dem Therapiearm mit schlackenreicher Ernährung zugeteilt, einer Ernährung, die in derartigen Situationen relativ kontraindiziert ist. Demnach gibt es keine spezifische »Crohn-Diät«. Dennoch sollte man den Patienten Richtlinien zur Ernährung geben. Diese orientieren sich an individuellen Unverträglichkeiten, einem eventuellen sekundären Malabsorptionssyndrom und entsprechenden Mangelzuständen sowie morphologischen Veränderungen im Gastrointestinaltrakt, die als Folge der Entzündungen oder Operationen entstanden sind. Besonders wichtig ist bei den oft untergewichtigen Patienten ein ausreichender kalorischer Gehalt der Ernährung (21, 30). Falls keine Kontraindikationen vorhanden sind, stellt auch für den Crohn-Patienten die faserreiche, vitaminhaltige und wenig raffinierte Kohlenhydrate enthaltende Mischkost wie für jedermann die beste Ernährung dar.

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Journal of Pediatric Gastroenterology and Nutrition
26:172–174 © February 1998 Lippincott–Raven Publishers, Philadelphia

Activated Protein C Resistance in Pediatric Inflammatory Bowel Disease

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ABSTRACT

Background: There is evidence for a hypercoagulable state in inflammatory bowel disease (IBD), and small vessel thrombosis has been identified in the bowel of patients with Crohn's disease, suggesting thrombosis as a possible etiologic factor. Activated protein C (APC) resistance is the most common inherited disorder leading to thrombosis and accounts for 30% to 40% of episodes of idiopathic venous thrombosis.

Methods: The prevalence of APC resistance was studied in 23 patients with IBD (17 with Crohn's disease, 6 with ulcerative colitis) and in 11 control subjects with recurrent abdominal pain or celiac disease, using an APC resistance screening method.

Results: One patient with Crohn's disease had a positive screen result, two patients (one with Crohn's, one with ulcerative colitis) had borderline results, and results in all of the control subjects were normal. One patient with Crohn's disease had a history of a thromboembolic event but had a normal screen result.

Conclusions: Activated protein C resistance does not seem to play a major role in the etiology of the hypercoagulable state in inflammatory bowel disease. ***JPGN 26:172–174, 1998.*** **Key Words:** Children—Crohn's disease—Hypercoagulability—Inflammatory bowel disease—Protein C resistance—Ulcerative colitis.

Inflammatory bowel disease, a term that refers to both Crohn's disease and ulcerative colitis, is a disorder of unknown origin. Current theories include defective immunoregulation, infections, and microvascular events in the gastrointestinal tract, coupled with a genetic predisposition. In recent years, several studies have presented evidence for a hypercoagulable state in inflammatory bowel disease (IBD) (1). This state includes arterial and venous thromboembolic phenomena, detected clinically and in autopsies (1–3). There is a paucity of IBD among patients with Von Willebrand's disease and hemophilia (4), and Dhillon et al. demonstrated microthrombi in normal tissue of patients with Crohn's disease and ulcerative colitis (5). Levels of some coagulation factors are elevated, but this is thought to be a secondary phenomenon. Platelet aggregation and increased thromboxane B_2 and β-thromboglobulin levels have been detected during active and inactive disease (6), and protein C levels are normal (7).

In 1994, Svensson and Dahlback described activated protein C (APC) resistance as a cause of venous thrombosis. Up to 40% of patients with recurrent venous thrombosis had APC resistance, compared with an occurrence of 3% to 7% in a control population, making it the most common identified procoagulant state (8,9).

The purpose of this study was to look for APC resistance in pediatric patients with IBD, to determine its possible role in the hypercoagulable state associated with IBD.

PATIENTS AND METHODS

Thirty-four children were enrolled in the study; 17 had Crohn's disease, 6 had ulcerative colitis, and 11 with recurrent abdominal pain or celiac disease served as control subjects. There were 16 boys and 7 girls in the IBD group, and 2 boys and 9 girls in the control group. There were significantly more girls in the control group ($p < 0.01$). The mean age in both groups was similar (IBD group 16.0 ± 3.1 years, control subjects 15.3 ± 2.1 years). One girl with Crohn's disease had a history of pulmonary embolism, but none of the other patients had a history suggestive of thromboembolic disease.

Blood was drawn into a test tube containing citrate used for prothrombin and activated prothrombin time. Centrifuged platelet-free frozen plasma was collected and frozen at −70°C. The APC resistance screen was performed using the commercial APC resistance kit developed by Chromogenix (Malmö, Sweden). Plasma, activated partial thromboplastin time reagent and calcium chloride were incubated until clot formation and a second analysis was performed with activated protein C. Clot

Received February 12, 1997; accepted July 9, 1997.

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detection was performed by ACL 2000 analyzer. In the normal population, the ratio of activated partial thromboplastin time, with and without a standardized amount of APC in the presence of calcium chloride, is above 2.5. In the affected population, the ratio is below 2 for homozygotes; 2 to 2.5 is considered borderline (10). The test was done during routine follow-up visits, irrespective of the disease activity or therapy.

Statistical Analysis

Statistical analysis was performed using Fisher's exact test.

RESULTS

One patient of 23 (4.3%) with Crohn's disease had a positive APC resistance screen ratio of less than 2, consistent with homozygous APC resistance, whereas none of the control subjects had an abnormal result. This difference was not statistically significant. In addition, 1 patient with Crohn's disease and 1 with ulcerative colitis had borderline ratios between 2 and 2.5.

DISCUSSION

Possible causes for thromboembolic disease and microvascular thrombosis in IBD include increased procoagulant factors, decreased fibrinolysis, increased platelet activity, or physical inactivity associated with disease. Increased clotting factors (2,5,8), increased fibrinogen, and decreased fibrinolysis have all been documented, but tend to correlate with disease activity (11–15), whereas many episodes occur during quiescence or after colectomy in the case of ulcerative colitis (16). Webberly et al. (16) found increased platelet aggregation, spontaneous and induced, in patients with IBD, and more relevantly, in seven of eight patients with a history of thromboembolism. Changes in platelet aggregation were independent of disease activity.

Protein C is a vitamin K-dependent zymogen to a serine protease with anticoagulant properties. When the coagulation system is activated, factor IX converts factor VIII to VIIIa, which leads to Va activation and conversion of prothrombin to thrombin. Formation of factors Va and VIIIa are the rate-limiting steps in thrombin formation. Thrombin binds to thrombomodulin, activating protein C. Activated Protein C cleaves and inactivates coagulant factors Va and VIIIa (9). Protein C deficiency leads to thromboembolism and necrotic ulcerations of tissues.

In 1994, Svensson and Dahlback described APC resistance as a cause of venous thrombosis (17). Resistance is caused by a mutation at nucleoside position 1641 in factor V, which is not degraded, causing continuous thrombin formation. Since that time, recurrent venous thrombosis was observed in up to 40% of patients who had APC resistance, as opposed to 3% of a control population, making it the most common type of inherited procoagulant state—at least 10 times more common than any other inherited defect of coagulation. This trait is autosomally dominant and may be silent. Combination with another procoagulant state may lead to thrombosis (8).

Only one of our 17 patients with Crohn's disease and none of the 6 with ulcerative colitis had an abnormal screen result that was compatible with homozygous APC resistance, and 2 others had borderline values. None of the control subjects had evidence of APC resistance, and the difference was not statistically significant. The only patient with a history of thromboembolism had a normal APC resistance screen result. The groups were not matched in relation to gender, but this is unlikely to be of importance in an autosomally recessive disease.

These findings do not support a causal role for APC resistance in the pathogenesis of IBD or in the hypercoagulable state associated with these diseases, although larger numbers may be needed for a definitive answer.

Gaffney et al. (18) reported the efficacy of intravenous heparin in refractory cases of ulcerative colitis, suggesting that modulation of the coagulable state may play a role in the etiology and future therapy of the disease. Further studies are needed to identify the causes of the hypercoagulable state and its therapeutic modification.

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Clinical Quiz

NASPGN Clinical Quiz Editor, Joseph F. Fitzgerald; and ESPGHAN Clinical Quiz Editor, *Riccardo Troncone

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Sandeep Gupta, Contributor

A 12-year-old boy was referred with a 4-week history of intermittent bright-red lower gastrointestinal tract bleeding. He denied abdominal pain, nausa, vomiting, diarrhea, and weight loss. He had not experienced fever, and had had no known infectious exposures. He described his stools as formed, nonpainful, and 1 to 2 per day. Blood was noted on the stool, in the toilet water, and on the tissue. His physical examination was completely normal. Specifically, he had no freckling of the lips of buccal mucosa, no skin lesions, and no bony or soft tissue tumors. Family history was negative for adenomatous polyposis coli, juvenile polyposis coli, and isolated polyps.

Complete colonoscopy was performed, and a polyp measuring 2 cm in greatest diameter on a 2 cm-long stalk was encountered 30 cm from the anus (Fig. 1). No other polyps, masses, or mucosal abnormalities were noted. The polyp was submitted for histologic studies. Diagnosis is:

A: Classical juvenile polyp
B: Mixed juvenile and adenomatous polyp
C: Peutz—Jegher polyp
D: Lymphoid polyp
E: Villus adenoma

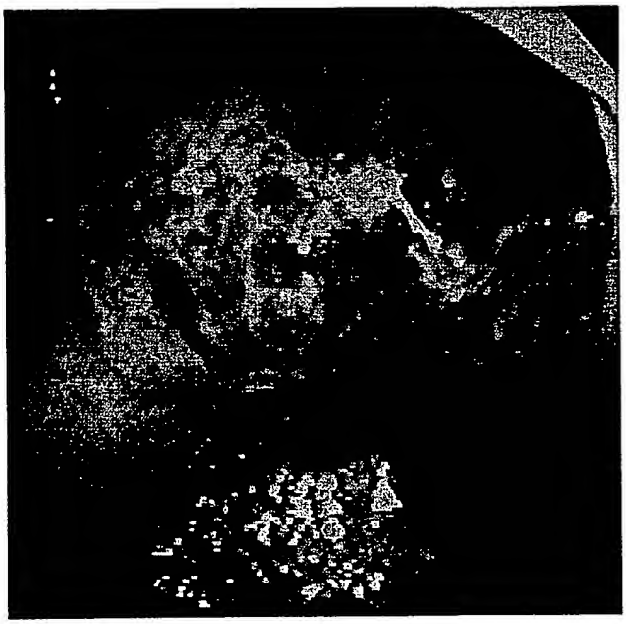

FIG. 1

ANSWER: see page 225.

Environmental Risk Factors in Inflammatory Bowel Disease

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Summary

Besides a genetic predisposition, a causal role of various environmental factors have been taken into consideration in the etiology of inflammatory bowel disease (IBD). The most consistent association of environmental factors so far identified is the association between non smoking and ulcerative colitis (UC) as well as between smoking and Crohn's disease (CD). Other factors such as oral contraceptives, refined sugar, perinatal events, childhood infections, microbial agents, and domestic hygiene have been found to be associated with ulcerative colitis and Crohn's disease but further evaluation is required to confirm the consistency and to define the strength of the association. Recent data also suggest that measles virus may persist in intestinal tissue and early exposure to the virus may be a risk factor for development of CD.
The further investigation of environmental factors on IBD and the explanation of their role is expected to open new avenues for basic scientific research and may lead to the development of a more rational approach to the prevention and treatment of IBD. The available data suggest that UC and CD are heterogeneous disorders of multifactorial etiology in which hereditary and environmental factors interact to produce the disease.

Key words: Breast feeding, Crohn's disease, diet, infections, measles, oral contraceptives, smoking, ulcerative colitis

Abbreviations: Crohn's disease (CD), Inflammatory bowel disease (IBD), Interleukin (IL), Leukotriene B4 (LTB4), Prostagladin E2 (PGE2), Relative risk (RR), Tumor necrosis factor (TNF), Ulcerative colitis (UC)

Introduction

It has been suggested that environmental factors may have an important role on the expression of inflammatory bowel disease (IBD). There are several observations supporting this hypothesis as the significant geographic variation in disease incidence, the low rate in concordance of monozygotic twins, the increased incidence in urban areas in comparison to the rural areas, and the increased incidence over too short time span. A number of environmental factors have been found either firmly associated with disease susceptibility or putatively related. These factors include the following:

- smoking
- oral contraceptive use
- dietary factors
- breast feeding and perinatal events
- measles virus
- childhood infections
- microbial agents
- domestic hygiene and
- miscellaneous: (toothpaste, appendectomy, tonsillectomy, blood transfusions, contact with animals, physical activity etc.)

This review will focus on the current understanding of the influence of these environmental factors in the pathogenesis of IBD. Further investigation of environmental factors and the explanation of their role is expected to open new avenues for basic scientific research and may lead to the development of a more rational approach to the prevention and treatment of IBD.

Smoking

Cigarette smoking is the most extensively studied and reported environmental factor in association with IBD. The first description by Harries et al (1) that smoking protects against ulcerative colitis (UC) stimulated a large series of epidemiological studies. This is due to the "strange" negative association between UC and smoking, as well as to the relatively ease of studying smoking habits in comparison to other environmental factors. On the other hand, the finding that smokers are at increased risk for developing Crohn's

Hepato-Gastroenterology 43 (1996) (Pages 381-393)

disease (CD) has led to the speculation that smoking may determine the type of IBD that develops in predisposed subjects (2). In addition, it has been reported that patients with IBD come from non-smoking households more often than healthy subjects do (1). In cases with positive family history of IBD there were high concordance rates between the type of IBD and smoking habits providing the suggestion that both smoking habits and genetics may be important factors in the pathogenesis of disease (3).

There have been several studies trying to resolve the effects of cigarette smoking on the pathogenesis and clinical course of IBD. This research has been extended to include the effects of passive smoking on IBD and has led to develop new experimental therapies such as nicotine in management of UC.

Ulcerative colitis

The most consistent association of environmental factors so far identified is the association between non smoking and ulcerative colitis. The most consistent observations which show that smoking protects from UC are:

- Decreased risk for UC in current smokers
- Increased risk in former smokers
- Possible dose-response relationship
- Hospitalization rate is lower in current smokers
- Pouchitis is more frequent in non-smokers
- Nicotine treatment improves UC

An abundance of reports from different areas show almost without exception a decreased risk of UC for current smokers **(Table 1).** It has been also demonstrated in the majority, but not in all of the studies, that former smokers are at increased risk of disease. Meta-analyses with the estimation of the better studies have been published. The strength of the association suggests that not smoking and UC are consistent with a causal relationship (4).

In a recent review of 56 studies, the influence of smoking in the epidemiology of UC has been examined (5). The sex distribution in UC has changed from a female predominance in earlier studies to a male predominance now. In contemporary pediatric studies, no such changes were seen. This suggested to the authors that the increase rate in men is related to the recent changes in the smoking habits. Diseases strongly associated with smoking as cardiovascular and respiratory system diseases (6) as well as lung cancer (7) have been found to be decreased among patients with UC in comparison with controls.

TABLE 1. SMOKING AND ULCERATIVE COLITIS: RELATIVE RISKS FOR CURRENT AND EX-SMOKERS IN COMPARISON TO NON-SMOKERS IN DIFFERENT AREAS.

Area	current smokers	ex-smokers
Cardiff, UK (1)	0.1	1.5
Nottingham, UK (121, 122)	0.2	2.1
Birmingham, UK (6)	0.2	0.7
Leeds, UK (59)	0.3	12.2
Oxford, UK (45)	0.8	1.2
Orebro, Sweden (12)	0.6	2.2
Stockholm, Sweden (20)	0.8	1.5
Milan, Italy (11)	0.6	2.6
Bari, Italy (123)	0.3	1.4
Boston, USA (10)	0.3	1.1
Baltimore, USA (124)	0.6	1.1
Washington, USA (13)	0.6	2.0
North Carolina, USA (8)	0.5	1.3
Chicago, USA (14)	0.1	1.2
Japan (63)	0.3	1.7

Childhood passive smoke exposure has been observed possibly influencing adult susceptibility to UC but the results of the existing studies are not consistent. Sandler et al (8) report that passive childhood exposure to environmental tobacco appear to decrease the disease risk in adulthood, but Lashner et al (9) observed the opposite, namely an increased risk of disease in adulthood. Their suggestion is that the mechanism in these cases is similar to the adult ex-smokers who develop UC.

Heavy smokers were found in several studies to have lower risk of UC than light smokers (10-12). However, no dose-response effect based on pack-years of smoking was found in other studies (13,14). The hospitalization rate of current smokers with UC was also found to be lower than of non-smokers, but colectomy rate in persons who smoked after disease onset and non-smokers however was similar (15). The risk of developing pouchitis after formation of an ileo-anal reservoir was found to be higher in non-smokers (16). Some patients with UC who resumed smoking had an improvement of symptoms (17) although no controlled study data are available. With the possibility that nicotine is the ingredient of tobacco smoke responsible for improvement, nicotine gum as well as transdermal nicotine have been used in the treatment of UC. In a recent controlled study (18) 17 of the 35

patients in the transdermal nicotine group had complete remission, in comparison with 9 of the 37 patients in the placebo group (P=0.03). Transdermal nicotine however, has been found ineffective as maintenance therapy for UC (19).

Crohn's disease

In contrast to the protective effect on ulcerative colitis, smoking is deleterious for Crohn's disease. This is supported by the following observations:

- Increased risk for CD in current smokers
- Increased recurrence rate in smokers
- Increased risk for operations and complications in smokers
- Association of smoking with small bowel disease
- Increased risk for CD in passive smoking of childhood
- Giving up of smoking leads to lower recurrence rate

The relative risk for CD is increased in smokers in comparison to the non-smokers as has been demonstrated by several studies **(Table 2).** Meta-analyses have shown that smokers are more than twice as likely to develop CD (4). Ex-smokers have also been found in some studies at increased risk but lower than current smokers. The relative risk of CD associated with smoking has been reported greater for women than for men with a threefold difference (20).

TABLE 2. SMOKING AND CROHN'S DISEASE: RELATIVE RISKS FOR CURRENT AND EX-SMOKERS IN COMPARISON TO NON-SMOKERS IN DIFFERENT AREAS.

Area	current smokers	ex-smokers
Cardiff, UK (125)	1.2	1.7
Oxford, UK (45)	1.8	0.8
Nottingham, UK (2)	3.5	
Nottingham, UK (122)	1.9	1.6
Orebro, Sweden (12)	2.0	1.9
Milan, Italy (11)	3.9	3.2
Essen, Germany (51)	3.0	
Chicago, USA (26)	3.7	

The risk for recurrence in patients with CD has been found to be significantly increased in smokers in comparison to the non-smokers (21). The risk for operations, reoperations, and complications was also increased in smokers compared to non-smokers (22). Moreover, patients with a high degree of life tobacco exposure and heavy smokers had small bowel disease more often than patients with lower life time exposure and light smokers. In the same study the proportion of patients with isolated colonic CD did not vary between non-smokers and smokers providing the suggestion that smoking does not protect from colonic CD (22). An association between cigarette smoking and small bowel CD, defined as small bowel disease plus combined small and large bowel disease, was also reported by three other studies (23-25). The patients with CD seem to be less likely to quit smoking than the controls (26). A recent study (27) shows that smoking is an independent prognostic risk factor for clinical, surgical, and endoscopic recurrence in CD. An intriguing finding was that ex-smokers had a risk of recurrence similar to non-smokers; giving up smoking soon after surgery was associated with a lower probability of recurrence supporting the view that it is highly worthwhile to advise CD patients to stop smoking.

Passive smoking in childhood has been found to be associated with increased risk to develop CD (9, 20). The association was stronger for CD than for UC and there was a dose-response effect. Heavy smoking, passive childhood smoke exposure, and Jewish heritage were independently associated with the clinical phenotype of fistulizing CD in a more recent study (28).

Pathogenetic mechanisms

The mechanisms by which smoking may be protective in UC and deleterious for CD remain unknown. Investigations of the influence of cigarette smoking on intestinal physiology, mucosal blood flow, mucus production and synthesis, intestinal permeability, and local immune function are in progress with the hope to provide a better insight into the ultimate pathophysiological initiation of IBD. So far the results of these investigations have shown that smoking:

- Increases colonic mucus production (29)
- Decreases intestinal permeability (30)
- Reduces production of PGE2 (31)
- Reduces rectal blood flow (32)
- Decreases colonic IgA and increases IgG production (33)
- Reduces the ratio of T-helper-inducer to T-suppressor cells (34)
- Increases TNF and IL-6 production (35)

These data provide therefore, some speculations on the mechanisms involved in the opposite association between UC and CD with smoking. In UC the colonic mucus is significantly reduced in comparison to healthy controls (36). The increase of mucus production as a consequence of smoking may lead to a restoration of a normal pattern of mucus and protection against UC. The decreased intestinal permeability with smoking may protect against UC by limiting exposure to harmful exogenous agents. Prostagladin E2 (PGE2) is increased in patients with active UC in comparison with healthy controls (37). The significant reduction of PGE2 in heavy smokers may protect them from UC.

Multifocal gastrointestinal infarction which is mediated by a chronic mesenteric vasculitis has been proposed as a pathogenic mechanism in CD (38,39). The event that smoking reduces blood flow in the rectum and increases the thrombotic tendency with associated vascular damage, could explain the involvement of smoking in Crohn's disease.

The finding that smoking decreases the colonic IgA and increases the IgG in vitro (33) provides the suggestion that smoking may interfere with the normal humoral immune defenses, and perhaps alter mucosal cell mediated defense mechanisms active in colonic mucosa (40). Systemically heavy smoking induces a reduced ratio of T-helper-inducer to T-suppressor cells (34) and increases TNF and IL-6 production in smokers in comparison to non smokers (35). However, data on IBD patients are still not available.

It has been suggested that nicotine improves UC through an effect on inflammatory mediators (31) or by changing adherent surface mucus in the colon (41). Subcutaneous administration of nicotine in rabbits changed the thickness of adherent rectal mucus with an inverse dose relationship and reduced rectal eicosanoids (42). Moreover, Madretsma et al (43) found that nicotine inhibits the production of IL-2 and TNF-a by peripheral blood and colon mucosal mononuclear cells, via a non-cholinergic nicotine receptor. These findings should stimulate further studies in IBD patients.

Oral Contraceptives

Several studies have shown a weak but consistent association between oral contraceptive use and both UC and CD. The pattern of the association usually is reported as increasing relative risk with increasing duration of use and decreasing risk with increasing interval since the last use which is consistent with a cause and effect relationship. The existing data about the association of oral contraceptives and IBD show that:

- The risk appears to be worse for CD than UC
- Thromboembolism and ischemia may be involved in the pathogenesis of the effect

Lesko et al (44) found that the relative risk (RR) for CD of oral contraceptives users was 1.9 (95% confidence intervals 1.0-3.5) in comparison to the women who had never used these drugs, with the risk to be higher for the users of more than 5 years (RR:8.0). Prospective studies by Vessey et al (45), Logan & Kay (46), and a case control study by Persson et al (47) have reported increases in CD risk among current oral contraceptive users although the differences did not reach statistical significance. Another case control study by Lashner et al (48) found no association between CD and current or former use of these medications. However, a recent study by Boyko et al (49) have shown an RR: 2.6 (95% CI 1.2-5.5) for CD and women who had used oral contraceptives for more than 6 years had the highest risk (RR: 5.1 95% CI 1.8-14.3). Sandler et al (50) found a higher CD risk among women smokers who used oral contraceptives, while Katchinski et al (51) concluded that the risk was higher among non smokers only.

The risk of recurrence of CD after surgery was also reported without significant difference in oral contraceptive users in comparison to non users (52).

In UC patients, the existing data are more conflicting. Case control and prospective studies found no association between UC risk and oral contraceptive use (46,50,53). However, in the study of Vessey et al (45) as well as in the recent case control study by Boyko et al (49) the RR for UC was 2.5 (95% CI 1.1-5.6) and 2.0 (1.2-3.3) respectively.

The mechanism whereby oral contraceptives may increase the risk for IBD remains unknown. Two explanations have been suggested. First that oral contraceptives, which are known to increase the risk of thromboembolic disorders, induce intestinal ischemia and trigger a chain of events that culminates in clinical IBD. The multifocal gastrointestinal infarctions found with innovative techniques (38,39) would suggest that in susceptible hosts use of oral contraceptives may induce CD or in CD patients it will aggregate the disease. The second suggestion is that oral contraceptives predispose to an illness that clinically resembles CD, that cannot be distinguished from it by non-invasive

diagnostic procedures and remits when oral contraceptives are discontinued (44).

Dietary Factors

Dietary factors have been implicated in inflammatory bowel disease but so far very few positive associations have been reported. However, there are reasons to suppose that diet could influence susceptibility to IBD. The dietary antigens are the majority of non-bacterial and non-self-antigens that are present in the gut, and antibodies against some dietary factors have been reported in both UC and CD. The significant geographic variations of the disease as well as the increase of the incidence since the second world war have encouraged speculations about the role of modern environmental factors, including the diet, in the pathogenesis of IBD. However, so far the information on diet and IBD is inconsistent although not unimportant.

The dietary factors which have studied in IBD are:

- refined sugar
- margarine
- coffee and alcohol
- cornflakes
- fruit and vegetables
- fish
- fast food and food additives

High consumption of refined sugar in association with CD has been reported at first by Martini and Brandes (54) and has subsequently been confirmed by others (55-58). However, the high consumption of refined sugar may be secondary to the development of CD as a consequence of increased energy which need the patients with CD. This is more clear in the study of Järnerot et al (57) in which the sugar consumption by CD patients increased with time providing the suggestion that the increased sugar intake was a consequence of the disease and not causal. Although it is known that smoking is positively associated with high sugar consumption, in two studies smoking and sugar intake were independent risk factors for CD (59,60) without however, to increase the relative risk in cases with combined exposure to them (60). The geographic and temporal variations of sugar intake were found to have no relationship with the variations of the incidence and the mortality of CD in different countries (61).

Margarine consumption has also increased over time and the suggestion of possible association with CD has been suggested. However, no significant correlation was discovered when temporal and geographic correlations were examined (61).

Coffee and alcohol use have been studied in association with UC (62). There was no evidence that coffee ingestion changes the UC risk. The consumption of alcohol was found to be associated with decreased risk of disease and the risk declined as daily alcohol consumption increased. These results suggest some protective effect of alcohol consumption on development of UC and was confirmed from a study in Japan (63), but two other studies report no association between alcohol intake and disease (64, 65).

Patients with CD were found to consume larger amounts of cornflakes than controls (66), but other studies failed to confirm this. Consumption of fruit and vegetables has been reported to be lower in IBD patients (58, 67), but this may a secondary phenomenon, due to difficulties in handling fiber containing foods. A controlled study of a fiber rich diet as maintenance treatment in UC failed to show any benefit (68).

The consumption of fish has also been suggested as a possible important dietary factor in IBD. The observation that in Japan, where consumption of fish is very high IBD is less common (69), has led to speculations about the role of fish oil and its substance omega-3 fatty acids in IBD. It has been suggested that the western diet is deficient in omega-3 fatty acids compared with the diet on which humans evolved and their genetic patterns were established (70). Omega-3 fatty acids can alter the arachidonic acid metabolism and may have antiinflammatory actions. Initial reports with the use of fish oil or omega-3 fatty acids as supplements in UC have shown disease improvement compared with placebo therapy (71, 72). Further investigations are needed.

Food additives, especially carrageenans, have been discussed in the etiology of CD. Carrageenans are common ingredients in sweets and were found to induce colitis in laboratory animals (73). However, ready to eat commercial puddings and desserts with potential carrageenan content were studied in IBD but no association was found (67). Frequent consumption of fast foods has also been indicated in increased risk of IBD (74), but exposed numbers were small; therefore confirmation is needed.

Breast Feeding and Perinatal Events

There is accumulating evidence indicating that events early in life, including infant feeding practices, can have long terms effects on health and disease.

Absence of breast feeding has been found in some studies to be a risk factor for the development of CD and UC later in childhood (75-77). However, other studies failed to confirm that (67, 78-80). Koletzko et al (76, 81) found that breast feeding exerts a protective effect only in CD but not in UC and they suggest that this difference is associated with the different etiology of these diseases. In a recent study breast feeding was negatively associated with both UC and CD with a relative risk point estimates around 0.5 and with evidence of duration dependent trends in both instances (77).

The finding of birth cohort effects for both UC and CD by a population-based epidemiological study in Sweden (78, 82) has led to the suggestion that factors related to events surrounding birth or during pregnancy could play a role in causing IBD. The risk of IBD has been found to be increased four times for those with perinatal health events, including prenatal and postnatal infections in the mother, and postnatal infections in the child. The hypothesis that has been proposed is that perinatal events, probably infectious, in genetically susceptible individuals can change their immune response so that, later in life, an immunologically stressful event precipitates the onset of either UC or CD (83).

"The measles story"

Latest epidemiological and basic scientific data provide preliminary evidence for persistent measles infection in the intestine of CD patients. In Sweden during the years 1945-1954, five measles epidemics have been reported. A statistically significant excess number of diagnosed CD patients (but not of UC) born up to 3 months after each measles epidemic was found in comparison to the expected number of patients (84). This increased incidence of CD among people born during measles epidemics has implicated measles virus as a potential component cause, especially when exposure occurs in utero or early after birth.

In two French families with high incidence of CD, ultrastructural evidence of paramyxovirus has been reported (85). Furthermore, persistent measles virus infection has been identified in intestinal tissue of CD patients by immunohistochemistry for measles nucleocapsid protein, in situ hybridisation for measles - genomic RNA, electron microscopy (86), as well as by immunogold electron microscopy (87). The IgM specific anti-measles antibodies were found increased in 78 % of the cases with CD and in 59% of the cases with UC (88). However, measles virus RNA by PCR amplification was not recognized in ileocolonic tissue of CD patients in another study (89).

Based on the hypothesis that the primary pathological abnormality in CD is in the mesenteric blood supply and the previously reviewed data, Wakefield et al (90) have proposed that CD is a chronic granulomatous vasculitis in reaction to a persistent infection with measles virus within the vascular endothelium. However, if measles virus plays a part in the development of CD, a decrease in the incidence of CD due to the introduction of measles vaccination could be anticipated, as it is the case in subacute sclerosing panencephalitis. In contrast, the incidence of CD is increasing. Measles vaccination has recently been reported as a possible risk factor for both CD and UC (91, 92). Although this study has been criticized for several limitations (93), it underscores the need for additional epidemiologic and basic scientific studies to determine whether the wild type or vaccine virus of measles may play a role in the development of IBD.

Childhood Infections

It has been postulated that childhood events that modulate immune function, e.g. infections, may be significant in the pathogenesis of IBD. Moreover, it has been suggested that infections may precipitate IBD by unmasking antigens which are present in the gut or by generating an immune response directed against cross reacting proteins in the mucosa. Except from the possible alteration of the gut regulatory function by infections, the use of antibiotics and nonsteroidal antiinflammatory drugs during infections may play an aetiologic role.

A variety of infections, including gastroenteritis, tonsillitis, ear and respiratory infections has been reported in increased frequency in IBD patients during childhood. In an international cooperative study (67) recurrent respiratory infections were significantly more frequent in IBD patients than in controls. The repetitive use of antibiotics was more common in CD patients in comparison to controls. Another study found the relative risk estimates associated with gastrointestinal infections during childhood 3.5 for CD and 3.8 for UC, while the corespondent with respiratory or urinary infections was 0.9 (79). However, an increased frequency of childhood infections and especially infections of the upper airway and oropharynx, among persons with CD has also been shown by a recent study (80). Moreover, the report of more infections from CD patients was usually associated with fre-

quent treatment with antibiotics and an early onset of the disease.

The postulation that CD is associated with inability to down-regulate the inflammation may lead to the suggestion that the excess of upper respiratory infections reflects an increased propensity for clinical expression which means that patients with CD may express symptomatic infections that in normal individuals remain symptomless.

Microbial Agents

The possibility of an infectious origin for CD, initially suggested when the disease was first described, has never been abandoned. Several candidates have been proposed and investigated but the majority of the results were negative. The possible role of mycobacterium paraturbeculosis in the pathogenesis of CD has been extensively examined. The presence of granulomas, the isolation of this mycobacteria from mucosa and mesenteric granulomas, and from mucosa and mesenteric lymph nodes (although also in cases of UC and controls) as well as some serological data and PCR findings suggest a role in the origin of CD (94). However, the failure to demonstrate conclusively this mycobacteria in CD tissue (low isolation rate), the variable results of serological studies, the negative results of antimycobacterial drug treatment and the absence of expression of mycobacterial heat shock proteins do not confirm this hypothesis (95).

A recent study (96) using innunocytochemical techniques in intestines and mesenteric lymph node specimens of 21 patients from families with high frequency of CD showed that 75% were positively labelled with the antibody to Listeria. In addition 57% of the cases contained the E. coli antigen, and 44% contained the streptococcal antigen. These authors have proposed Listeria, Escherichia coli and Streptococcus spp as putative pathogenetic microorganisms in CD. However, the specificity and significance of these findings remain to be determined.

"Sheltered Child" And Domestic Hygiene

One of the theories for the explanation of the increase of the incidence of IBD and especially for CD concerns the "sheltered child" hypothesis. This hypothesis has been suggested initially for Hodgkin's disease that is caused by the delayed exposure to the Ebstein-Barr virus. According to this hypothesis when the child is oversheltered and overprotected, the contact with common agents happens at a later age. This delayed exposure could trigger an inappropriate (immunologic) response, which then renders the bowel susceptible to disease (67).

Gilat et al (67) failed to confirm the "sheltered child" hypothesis for IBD patients with examination of variable individual and grouped criteria. However, a recent study (97) comparing the housing of patients and controls in early childhood, found that CD was significantly more common in subjects whose first houses had a hot water tap and separate bathroom. In contrast UC showed no clear relation to household amenities. It has been suggested by the authors that CD is initiated by an enteric infection and improved hygiene from provision of better household amenities has limited the exposure of infants to enteric organisms that program the immune system of the gut, thereby rendering the bowel more susceptible to triggering infection later in life. This attractive hypothesis may explain the inability, so far to find a specific infectious agent in the gut with inflammation and the much higher prevalence of IBD in the developed as compared to the developing countries.

Another parameter that has been used in the hypothesis of "sheltered child" is the family composition of the patients. The multicentric study of Gilat et al (67) has shown that CD patients were more likely to be last born in comparison to the controls. Persson et al (79) have found an increased risk of both CD and UC among the subjects without siblings with stronger association in men. In the cases with siblings, those with older and younger siblings had a decreased relative risk compared to those with only younger or older siblings.

Miscellaneous

Appendectomy

Recent studies have shown that patients with UC rarely underwent appendectomy before the disease manifestation (67, 80, 97-100). The relative risk of developing or having UC found significantly lower in subjects who have undergone appendectomy than in subjects whose appendix was intact (98, 99), and this association was stronger than between smoking and UC (99). Another recent study however, failed to confirm this observation (101). There are two suggestions about the possible association between appendectomy and UC. The first one is that patients predisposed to UC are less prone to suffer from acute appendicitis due to coexistent factors such as altered intestinal motility, reduced fecalith formation, and mucin abnormalities. The second and most likely explanation is

that appendectomy is a protective factor against UC. The appendix is an important part of the gut-associated lymphoid system, together with Peyer's patches and tonsils and may be that the resection of the appendix influences the balance between ileocolonic helper and suppressor function and in that manner protects against UC (98). Although this inverse association seems to be specific, larger observational studies are needed to confirm its consistency and to define better its strength.

Tonsillectomy

Initial studies failed to find any association of tonsillectomy and IBD (67). However, in a recent study, the incidence of tonsillectomy has been found increased in CD patients and has led to the suggestion that increased frequency of tonsillitis is an early harbinger of more generalized dysfunction in gut lymphoid tissue (80). This association was not confirmed in two more recent studies (99, 101). On the other hand, it has been reported that tonsillectomy influences the clinical course of CD, conditioning a lower incidence of relapses, confinement of disease to the ileum, and a lesser extent and severity of disease (102).

Blood transfusions

The influence of perioperative blood transfusions on the recurrence of CD has been investigated in several studies. In two of them (103, 104) a significant lower recurrence rate has been found in the group of the transfused patients in comparison to the non transfused. However, in a larger study (105) there was an initial delay in recurrence in the transfused group but not statistically significant. A more recent study has shown no protective effect of blood transfusion for the overall number of patients, but a beneficial effect especially in parous females (106). Blood transfusions seems that have immunosuppressive effects (107) and may be this is the explanation of these observations but their role in IBD patients has not been completely explored.

Contact with animals

Gilat et al (67) found that patients with UC had more frequently a rodent as a pet in comparison to healthy controls. Another study also reports that keeping a bird as a pet was associated with an increased relative risk for CD among women (79). However, these data are not enough, and further studies are needed about the possible association of IBD with the contact with animals.

Physical activity

A striking finding of a recent study (79) was that high physical activity seemed to decrease the risk for CD, even after adjusting for other risk indicators. Comparison of different occupations (108) has also shown that outdoor workers who do more physically active work, had a lower risk of IBD. So far there is not a clear explanation about this association.

Hypothetical factors, toothpaste and xenobiotic metabolism

A hypothesis has been put forward about the possible role of toothpaste in the pathogenesis of CD (109). According to this hypothesis an ingredient in swallowed toothpaste may damage the gut and lead to CD. Toothpaste contains abrasives, foaming agents, flavoring mixtures, humectants, and thickening agents or binders and one or more of them may be responsible for the enteric lesions. The concurrence of toothpaste use with increasing incidence of CD and the similarity of CD lesions with lesions produced by material such as chalk point to this hypothesis, but so far there are no clinical or experimental data to support this.

Another recent hypothesis (110) proposes that UC is caused by a reactive xenobiotic metabolite (an exogenous agent, such as an environmental chemical not present in the body) which is excreted in bile and activated during its passage through the colon. The result is the damage of colonic epithelial barrier and the exposure of the mucosal immune system to luminal contents. Although the author's opinion is that this hypothesis is consistent with most of the current knowledge of pathogenesis of UC, the future test will define its strength.

Contribution Of Environmental And Genetic Factors In IBD

There is strong evidence that genetic and environmental factors play a significant role in the pathogenesis of ulcerative colitis and Crohn's disease. The observations supporting hereditary factors are increased familial incidence, higher rates in monozygotic versus dizygotic twins and higher rates in first degree relatives versus spouses. The variant frequencies found in different ethnic groups and the association of IBD with genetic syndromes and diseases with known genetic predisposition also support this hypothesis (111, 112). However, the search for a specific genetic marker in IBD has been disappointing. So far only a weak association of HLA DR2 (113) and

recently of allele 2 of IL-1ra (114, 115) with UC have been reported.

On the other hand, the epidemiological studies of IBD have provided information on the role of environmental factors in IBD. Both CD and UC are more common in the developed countries of Europe and North America than in Africa, Asia and South America. IBD also appears more common in urban than in rural areas. The incidence of UC has remained stable but the incidence of CD has increased significantly over too short time span and in some areas is now more than UC (116). Studies of different ethnic groups have shown that migrants in developed countries appear to acquire higher rates of IBD (117) and the rates among Jews are high but vary by country of origin. Studies on twins with IBD have shown (118, 119) that not all the monozygotic twins are disease concordant indicating the importance of the environmental factors. Moreover, giving up smoking in healthy monozygotic twins of IBD patients was not enough to develop UC.

Several environmental factors have been studied in association with IBD. So far the most consistent association is with smoking. The opposite associations of UC and CD have become so interesting that it is expected that the account of these opposite associations may unlock the door to a fuller understanding of cause and pathogenesis of IBD (120). Other factors as oral contraceptives, refined sugar, perinatal events, microbial agents, and domestic hygiene may play a role in the etiology of IBD but further evaluation is required to confirm their consistency and to define their strength.

In conclusion the available data suggest that ulcerative colitis and Crohn's disease are heterogeneous disorders of multifactorial etiology in which hereditary and environmental factors interact to produce the disease. The finding of markers of genetic and environmental risk may will lead to a better understanding of the natural history of IBD and to identify useful treatment options.

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Acknowledgements

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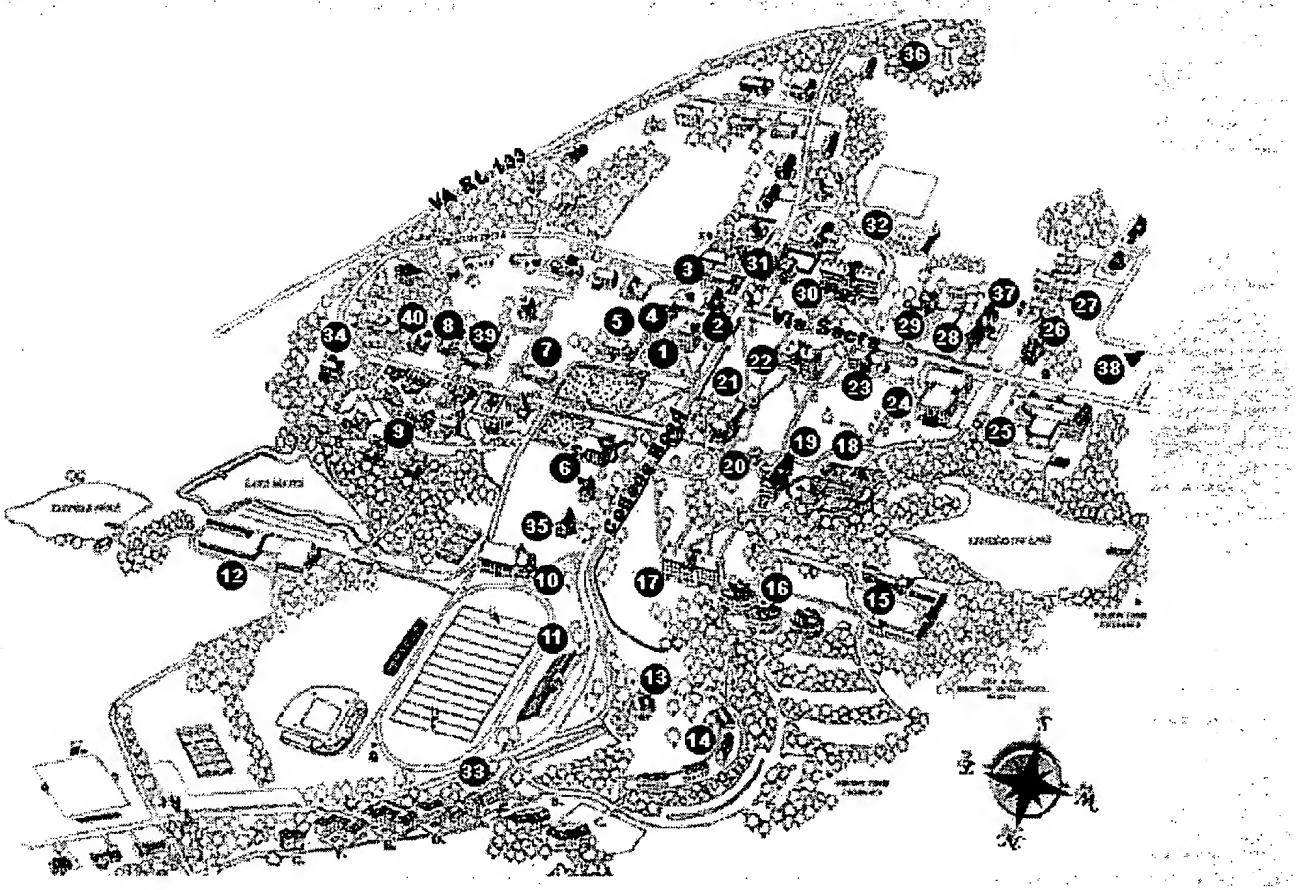
Ed Devlin's Home Page

Home | Courses | Research | Personal

I have been a member of the Biology Department at Hampden-Sydney College since 1990. My family, pets and I live on the HSC campus. During a recent sabbatical I spent some time updating the material on this webpage. You can go the the links above to get some information on my HSC courses, research and personal interests.



Hampden-Sydney College (what do all those numbers mean anyway?)

CV

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2. DEGREES AND EDUCATION

Ph.D. 1982 North Dakota State University, Fargo, North Dakota. Zoology (Embryology)

M.A. 1978 Bemidji State University, Bemidji, Minnesota. Biology (Botany)

B.S. 1972 University of Maryland, College Park, Maryland. Microbiology (Zoology)

3. CURRENT EMPLOYMENT RECORD

1990-Present Elliott Endowed Professor of Biology, Hampden-Sydney College.

4. PRIOR EMPLOYMENT RECORD

1985-1990 Assistant Professor of Biology, College of St. Catherine, St. Paul Minnesota. Courses taught: Embryology, Histology, majors General Biology sequence, Comparative Animal Physiology and non-majors Introductory Biology sequence.

1985 Lecturer, Department of Pathology, University of Washington Medical School, Seattle, Washington. Course taught: Introductory Histology and Pathology.

1983-1985 Postdoctoral Senior Research Fellow, National Institute of Health Fellowship in Environmental Pathology, Pathology Department, University of Washington Medical School.

1982 Instructor, Zoology department, North Dakota State University, Fargo, North Dakota. Course taught: Anatomy and Physiology.

1979 Visiting Scientist, U.S. Environmental Protection Agency Water Quality Laboratory, Duluth, Minnesota. Involved in toxicity testing with *Pimephales promelas* (fathead minnow).

5. COURSES TAUGHT AT HAMPDEN-SYDNEY

Introductory Biology, Biology 101-102, Principles of Biology, Biology 110
Introductory Biology Laboratory 151-152
Cell Biology, Biology 301, Genetics Biology 201
Histology, Biology 302
Developmental Biology, Biology 402

7. DEVELOPMENT EFFORTS AT HAMPDEN-SYDNEY

2002 NSF CCLI grant for a ESEM/EDS system (200,000.00), grant under review

1999 Howard Hughes Medical Institute Grant for upgrading facilities and programs in the Biology Department ($1,000.000.00) not funded.

1995 Howard Hughes Medical Institute Grant for upgrading facilities and programs in the Biology, Department (1,000,000.00), not funded.

8. PUBLICATIONS OR OTHER SCHOLARLY OR PROFESSIONAL PRODUCTS

Gottfried, I.S., H.R. Nash, E.W. Devlin. 1999. Presence of novel forms in the gut of inflammatory bowel disease patients. American Journal of Gastroenterology, 94(2):537-539.

Devlin, E.W and Clary, B. 1998. In Vitro toxicity of methyl mercury to fathead minnow cells. Bull. Environ. Contam. Toxicol. 61(4): 527-533.

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Devlin, E.W. and N.K. Mottet. 1992. Comparative methyl mercury toxicity on a mammalian and teleostean cell line. In Vitro Toxicology, 5(2) :83-91.

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Devlin, E.W. and N.K. Mottet. 1984. Effects of methyl mercury on early Salmonid development. Teratology 29(2):26-27.

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Devlin, E.W. 1982. Flora of forest clearings created by logging in Beltrami County, Minnesota. Prairie Naturalists. 14(2):46-50.

10. COLLEGE SERVICE

Administrative duties: (has served or is serving on) Faculty Affairs, Academic Affairs, Student Affairs, Appeals, Housing, Elliott Selection (chair), Human Research Review and Recycling Planning Committees, Chair of Biology Department, Faculty Advisor for the Student Environmental Action Coalition (SEAC) and Team Hampden-Sydney (cycling club) , freshman advisor, transfer advisor, OCTA instructor.

11. HONORS OR AWARDS

2001 Awarded a National Science Foundation Research Opportunities Award grant. Early cleavage in Xenopus development. Grant to support sabbatical research at Oregon Health Sciences University summer of 2001.

1996 Awarded an Elliot Endowed Professorship at Hampden Sydney College.

1995 Awarded an National Science Foundation Instrumentation Grant to introduce quantitative fluorescence light microscopy in the undergraduate curriculum , biology department, HSC (50,000.00).

1995 Awarded a National Science Foundation Research Opportunities Award grant. Role of retinoic acid in the anterior/posterior axis formation in *Xenopus* embryos to support sabbatical research at the University of Virginia 1995.

1994 Universal Research Fellow, Woods Hole Marine Biological Laboratory. Awarded a competitive Summer Fellowship to develop techniques and procedures in analytical and quantitative light microscopy for calcium ratio imaging in teleostean cells.

1992 Awarded a National Science Foundation Instrumentation Grant to establish computer mediated laboratories in physiology, biology department HSC ($50,000.00).

1990 Awarded a National Institutes of Health AREA Grant to study the comparative *in vitro* and *in vivo* toxicity of methyl mercury, College of St. Catherine ($70,000.00).

1988 Awarded a National Science Foundation Instrumentation Grant establish computer-mediated laboratories in physiology, College of St. Catherine ($32,000.00).

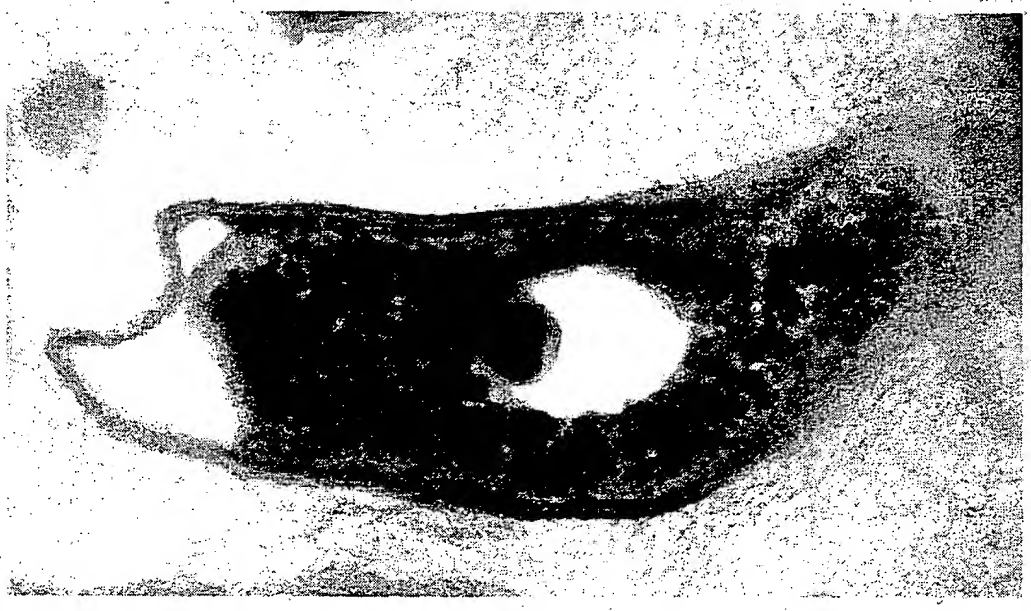
1987 The College of St. Catherine Faculty Teaching Award for Outstanding Achievement as Educator "teacher of the year" award, $2,500.00 cash prize).

1986 Awarded a Research grant from the Bush Foundation to set up an aquatic toxicology laboratory at the College of St. Catherine, St. Paul, MN.

1983 Awarded a National Institutes of Health Post-Doctoral Fellowship in Environmental Pathology to study *in vitro* and *in vivo* effects of heavy metals on development. University of Washington Medical School, Seattle, WA.

Inflammatory Bowel Disease

Home



Above is an electron micrograph I recently took of one of the organisms found in the tissue space at the site of a lesion in the colon of a patient with inflammatory bowel disease (IBD). Our current work involves analyzing tissue samples of IBD patients to identify the organisms present. Below is a portion of a paper we wrote on our preliminary findings.

PRESENCE OF NOVEL FORMS IN THE COLON

OF INFLAMMATORY BOWEL DISEASE PATIENTS

ABSTRACT

Objective: A number of theories have been proposed to describe the causative agent in inflammatory bowel disease (IBD). However the etiology of IBD remains unknown. The present study was designed to characterize the appearance and the frequency occurrence of novel forms identified in the colon of a group of IBD patients. ***Methods***: Biopsies from 81 colonoscopy patients were obtained and stained with a variety of stains including H&E and modified methenamine silver stain (GMS) for light microscopy. Stained specimens were analyzed under both normal and epifluorescent illumination. ***Results***: Non-fluorescent GMS-stained forms were identified in 75% (± 11 95% CI) of nonspecific colitis patients: 70% (± 28 95% CI) of Crohn's patients: 100 % of ulcerative colitis patients and none were present in benign disease patients. Fluorescent GMS-stained forms were in 66% (± 12 95% CI) of the patients diagnosed with non-specific colitis: in 50% (± 31 95% CI) of the patients diagnosed with Crohn's disease and in 75% (± 42 95% CI) of the patients diagnosed with ulcerative colitis and no examples of fluorescent GMS-stained forms were found in patients identified with benign disease. The appearance of the novel forms were characterized under light and electron microscopy. ***Conclusions:*** Novel forms have been identified in the colon of a high proportion of IBD patients.

Inflammatory bowel disease (IBD) is a group of diseases which includes Crohn's disease, ulcerative colitis, and may include nonspecific colitis. Clinical, epidemiological, and experimental studies have been used to attempt to elucidate the etiology of IBD (1, 2). A number of factors, individually or in combination, have been implicated as causative agents for IBD. These factors include genetic (3), microbial (4), viral (5), immunological (6), nutritional (7), thrombosis (8) and environmental (9) agents as possible

causative entities. For example, *Mycobacterium paratuberculosis* (10) , *Pseudomonas maltophilia* (11) and viruses (5) have been identified in the gut of IBD patients and hence implicated as etiological agents. A key feature associated with IBD is the activation of macrophages as well as other immune system activity. This involvement of the immune system has resulted in a number of proposed immunological and autoimmune models of IBD pathogenesis (12, 13). An increase in the incidence of IBD has also been reported in several of industrial countries which has lead to the suggestion that environmental (abiotic) agents may be responsible for IBD (14). In spite of the many proposed mechanisms and increasing interest in IBD, none of these studies have been able to demonstrate a clear cause and effect relationship and the etiology of IBD remains unknown.

In the present study, colonoscopy was performed and biopsies were taken from 81 patients with a diagnosis of diarrhea or lower GI bleeding during 1997 and 1998. Biopsy samples were fixed in neutral buffered formalin, embedded in paraffin, sectioned at 4um stained and mounted. A variety of different stains for light microscopy were applied including Periodic Acid Schiff (PAS), Feulgen, hematoxylin-eosin (H&E), acid fast stain, and modified methenamine silver (GMS) stain (Richard Allen Scientific-Chromaview). Samples for light microscopy observation were observed and photographed using an Olympus Vanox and IX70 inverted fluorescence microscopes with normal and 420 nm cube. Images were processed using Metamorph/Metaflour image analysis software (Universal Imaging). Non-fluorescent GMS-stained forms were identified and scored under normal light, fluorescent GMS-stained forms were observed and scored under epifluorescent illumination. Confidence limits for the population proportion were calculated using standard procedures (15).

Based on a review of the specimens stained with H&E, a diagnosis of nonspecific colitis was made in 43 patients; Crohn's disease was diagnosed in 10 patients; ulcerative colitis was diagnosed in 3 patients; and non-inflammatory disease was diagnosed in 5 patients. H&E and acid fast stained specimens did not reveal any novel forms. Specimens were then stained and observed under normal illumination with modified methenamine silver stain. Under these conditions a number polymorphic forms were observed that initially were thought to be a microsporidia-like pathogen except that they were negative for acid fast stain and expressed a different morphology. The non-fluorescent forms were found both individually or in groups in epithelial, lamina propia and stromal layers (see figure 1). These non-fluorescent forms were identified in 46 of the 61 nonspecific colitis patients, 7 of the 10 Crohn's patients, 4 of the 4 ulcerative colitis patients and none of the benign disease patients.

Under epifluorescent illumination with GMS stain, another form, often associated with regions of ulceration, was identified (see figure 1). These fluorescent forms were not colocalized with the GMS-stained forms we observed with normal illumination. The fluorescent forms were identified in 40 of the 61 nonspecific colitis patients, 5 of the 10 Crohn's patients, 3 of the 4 ulcerative colitis patients and again, none of the benign disease patients (see figure 2). Confidence intervals were calculated for the proportion of patients with both the non-fluorescent and fluorescent forms. In all cases the proportion of patients with non-fluorescent and fluorescent forms are greater than would be expected from random chance 95% of the time (see table 1).

Granuloma were present in a number of the IBD patients. In patients with granuloma, the non-fluorescent and fluorescent forms appear to be localized in the peripheral edges of the granuloma. Granuloma were found in 6 of the 43 nonspecific colitis patients, in 8 of the 10 Crohn's Disease patients, in 1 of the 3 ulcerative colitis patients and none were found in the benign disease patients.

The etiology of IBD has been extensively investigated and several models have been proposed with no known causal relationship having been established. In the present study, novel forms were identified from biopsies of the colon and the terminal ileum taken from IBD patients. These patients clinically expressed nonspecific chronic cellular inflammation of the colon in one of three different pathologic forms: nonspecific colitis, Crohn's disease, and ulcerative colitis. Some patients had evidence of either granulomata, active inflammation, ulcers with red halos, evidence of active colitis, or classic-appearing Crohn's disease with associated symptoms of diarrhea, abdominal pain or GI bleeding. In the present study previously unreported forms visible with GMS staining were found in a high percentage of these IBD patients. Work in our laboratory is currently underway to characterize the ultrastructure of these novel forms.

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Frequency of Forms in Patients With Different Diagnosis

Diagnosis	Number Patients	Non-Fluorescent #	Non-Fluorescent	Fluorescent #	Fluorescent	Granuloma
Nonspecific Colitis	61	46	0.75 ± 0.11	40	0.66 ± 0.12	6
Crohn's	10	7	0.70 ± 0.28	5	0.50 ± 0.31	8
Ulcerative Colitis	4	4	1.00	3	0.75 ± 0.42	1
Benign Disease	6	0	0	0	0	0
Totals	81	57	0.70 ± 0.10	48	0.59 ± 0.11	15